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VOLUME 37, No. 4 DECEMBER 1960

THE CO-ORDINATION OF SWIMMERET MOVEMENTS IN THE CRAYFISH, *PROCAMBARUS CLARKII* (GIRARD)

BY G. M. HUGHES* AND C. A. G. WIERSMA

Biology Division, California Institute of Technology, Pasadena, California†

(*Received* 25 *February* 1960)

Many Crustacea are characterized by the use of their appendages for swimming and feeding. In the lower Crustacea both the thoracic and abdominal appendages move for these purposes in a typical metachronal rhythm. The similar rhythmical movements found in the abdominal appendages of the crayfish (Hughes & Hickey, in preparation) are most probably based on similar nervous mechanisms, but here their function is not so clear. It is probably connected with the increased need for aeration when the animal burrows in mud and, in the female when in 'berry', with the oxygen supply for the developing larvae. The swimmeret movements provide very good material for a study of the general problem of the mechanisms of nervous control of rhythmic limb movements. Few observations have been made on Crustacea from this point of view and the crayfish is very suited for this purpose as considerable information is now available concerning its nervous system (Hughes & Wiersma, 1960; Wiersma, 1958; Wiersma & Hughes, 1961). The preparations also proved of interest as in some the influence of stimulating fibres descending from the brain was shown. Clear evidence for the presence of pathways with excitatory or inhibitory effects on the discharge of motoneurones in the abdominal ganglia has been obtained.

MATERIALS AND METHODS

The crayfish, *Procambarus clarkii* (Girard), was used throughout the experiments, for many of which females were preferred because five pairs of swimmerets could be observed during the movements. In the male the first two pairs are modified as secondary sexual organs and usually do not take any noticeable part in the rhythmic movements. Movement of the intact swimmerets interfered with recording by shorting the electrodes, especially in the female, and this was largely prevented by removing most of the exopodites and endopodites. Rhythmic movements continued normally following such operations and with little apparent change in frequency. The methods of dissection and preparation of specific motor-fibre bundles in the commissure have been described by Wiersma (1952), and the preparation of primary sensory fibres and interneurones located in the abdominal cord involved in swimmeret movements by Hughes & Wiersma (1960). In all experiments the preparation was bathed continuously in van Harreveld's solution. Recordings were made from

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* On leave of absence from the Department of Zoology, Cambridge, England.

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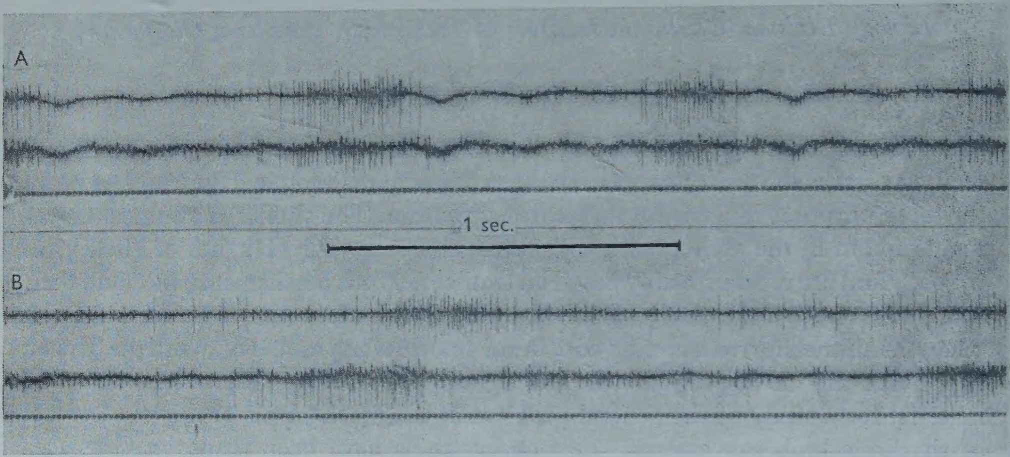
the first and second roots or from the connectives between individual abdominal ganglia using micromanipulated platinum electrodes, Tektronix 112 pre-amplifiers and a Dumont 322 oscilloscope. Ciné films were made using a Ciné Kodak Special 16 mm. camera at speeds of 16–64 frames/sec., for the loan of which we are greatly indebted to Prof. Albert Tyler.



Text-fig. 1. *Procambarus clarkii.* Isolated abdomen preparation. Electrical recordings of efferent discharges in first roots of the abdominal ganglia. In all cases the roots are cut distal to the electrode. A. Discharges in the first roots of the 5th ganglion. B. Discharges in the ipsilateral first roots of the 4th (upper) and 5th (lower) ganglia. The main bursts are normally associated with retraction of the swimmeret but subsidiary bursts associated with the protractor movements are visible in some cases (B). Time marker: 60 cyc./sec. and 1 sec.

RESULTS

(1) *Activity in the first roots of the isolated abdomen preparation*

When the abdomen of the crayfish is removed from the rest of the body the swimmerets often immediately start well-co-ordinated rhythmical movements which are essentially the same as occur in the intact animal. This is probably caused by the cutting of descending pathways from the brain, of which there are both excitatory and inhibitory ones present as will be discussed below. It was observed that the isolated preparation is more prone to give these movements than are preparations in which the whole C.N.S. is kept intact. The frequency of the movements is between 30 and 60/min. All swimmerets usually become active but sometimes the movements may be restricted to one or two pairs, or to a varying number of swimmerets on one side. An electrode placed under any first root readily picks up rhythmic bursts of impulses which are synchronous with the movements of the swimmeret which it innervates. The bursts persist when the nerve is cut distal to the electrode so that the recorded impulses are then entirely efferent (Text-fig. 1). These bursts are composed of several units and their timing shows a more or less well-defined pattern, those associated with retraction and protraction of the swimmerets being the most easily distinguished. A more detailed pattern is evident, however, and appears to depend on the part of the movement cycle with which a given unit is associated. The main discharge is always connected with retraction, at which time both the number of units involved and the overall frequency are highest. By splitting the first roots, as carried out in some preparations, it has been possible to obtain single unit discharges. In such preparations it is clear that the activities in the individual units grade one into the other rather than that there are distinct instants at which one unit becomes active and another inactive. This agrees with observations on vertebrate motor patterns under similar circumstances (e.g. Weiss, 1941).

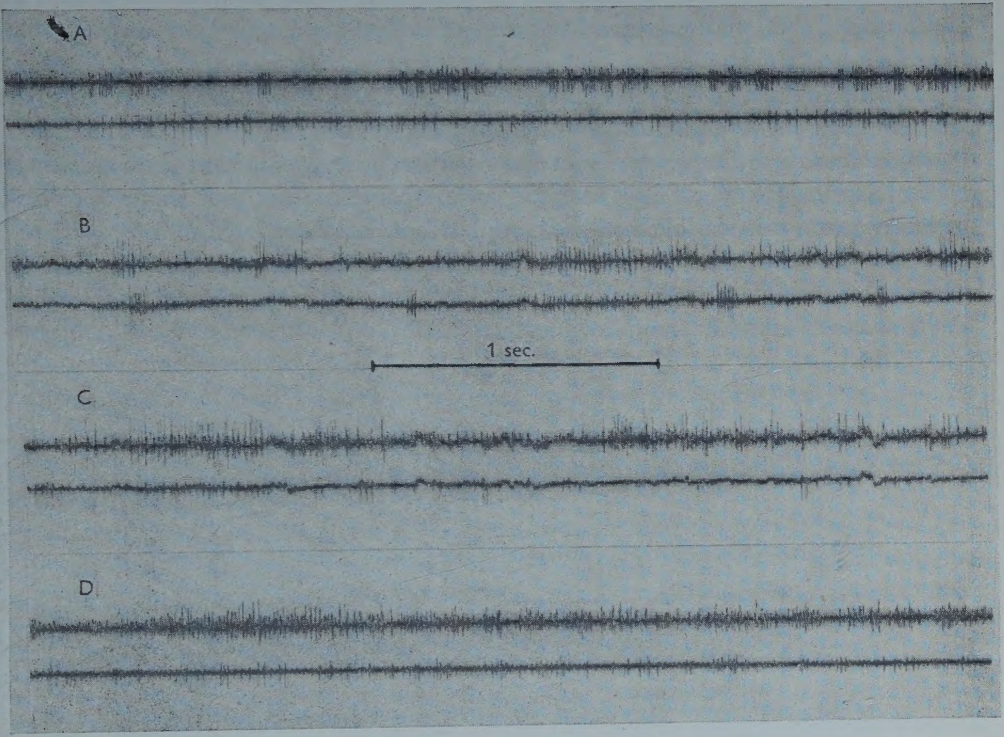
Normally the two swimmerets of a segment move together and the discharges recorded in the two first roots of a single ganglion show this expected synchrony, which persists when both roots are cut distally (Text-fig. 1 A). That this type of co-ordination is not rigid is indicated by the fact that individual swimmerets quite often show movements independent of the contralateral one. It seems possible for the co-ordination to range from a very rigid synchrony, accompanied by very similar nervous discharges in the two roots, to one of almost complete independence. Simultaneous recordings made from first roots of different segments on the same side clearly show the phase relationships between the segments even when the nerve is cut distal to the electrode (Text-fig. 1 B). The 5th swimmerets nearly always move first and are followed by the others as the wave of excitation spreads forwards. Again, there is no absolute rigidity about the phasing at certain times as is shown, for instance, in Pl. 16, A, which is taken from part of a record in which the 5th swimmerets showed five cycles and the 3rd only three, but some indication of an abortive burst can be recognized in the root of the 3rd ganglion. The overall findings suggest that each swimmeret has a centre of its own which is capable of controlling its rhythmic movements and that the coupling of this centre with those of neighbouring ones is not constant but varies in degree and at times can drop below an effective level. Co-ordination between swimmerets on the same side generally seems to be more persistent than that between pairs of the same segment. This preponderance of the ipsilateral co-ordination is also well known in other arthropods, for example, insects (Hughes, 1952).

(2) *Recordings from the connectives between two abdominal ganglia*

During some investigations into the function of nerve fibres in the connectives of the abdominal cord, several fibres were encountered which probably play a role in the co-ordination of the beat of the swimmerets. Of first importance is the direct evidence that has been obtained for proprioceptors which detect movements of their basal joints. These are primary sensory fibres which ascend at least across two ganglia (e.g. recording between the 3rd and 4th ganglion, fibres are present which fire when joints of the 5th swimmeret are moved). Each of the joint fibres is usually activated by one particular type of movement, for instance, either by maximal protraction or by retraction, and other fibres have been observed which respond only during separation of the exopodite from the endopodite, as occurs during the normal forward movement of the active swimmeret. Interganglionic fibres of another type encountered in the dorsal part of the connective have been described as 'activity' fibres. These fibres fire rhythmically in bursts (Pl. 15, fig. 1) when the swimmerets are active, but they are not excited by passive movements of the swimmerets. It seems possible that these recordings are from special fibres concerned in the operation of the basal joint sense organs similar to those described by Alexandrowicz & Whitear (1957), and Alexandrowicz (1958) for the basal joints of the thoracic appendages of various Crustacea. Such fibres would be connected directly to muscle fibres and could fire especially when this small muscle bundle contracts. It is of interest to note that this type of fibre for the 5th swimmeret was, in contrast to the previous ones, also present in the cord when investigated at the level between the 2nd and 3rd ganglia. Another possibility is that the bursts occur in collaterals of the efferent fibres to the swimmeret. In this way they would transmit a sort of 'efference copy' (von Holst & Mittelstaedt, 1950) to neighbouring ganglia. There is the additional complication that bursts also occur in interneurones several of which are known to be excited by joint movements of more than one swimmeret. The occasional presence of bursts of impulses without any swimmeret movements (Pl. 15, fig. 1C) is suggestive perhaps of an interneurone.

(3) *Progressive isolation of the abdominal cord*

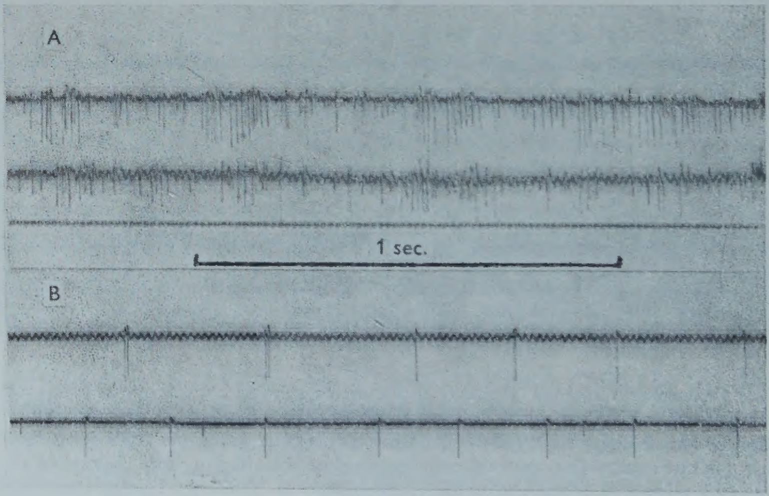
As has been described above, swimmerets of the isolated abdomen show a well-co-ordinated rhythm, and first roots show bursts of impulses which are in the correct phase of the rhythm of movements when the nerve is cut distally. The timing and general pattern of the periodic motor discharges is the same as when the nerve is intact. This offers an opportunity to study the extent to which the timing and nature of first root discharges depend upon the rhythmic inflow from the other ganglia and from the peripheral sense organs. It was found in the first place that cutting any or all second roots of all abdominal ganglia had no influence on the discharges. This might be expected since they are without sensory fibres coming from the swimmerets. Additional cutting of the third roots which are motor to the abdominal flexors did not alter the picture. More significant was the fact that severing the cord between the 5th and 6th ganglia again had no effect by itself or in combination with other cuts. This not only removed the inflow from the many roots of the 6th ganglion, but also excluded the not unlikely possibility of it being the site of a centre co-ordinating the swimmeret movements. Further isolation of the cord from sensory inflow was effected by cutting first roots, recordings being made from two of them, usually of the 3rd and 5th ganglia on the same side. With the progressive isolation the discharges show increasing changes but a clear rhythm persisted even when only a single root was intact. Pl. 16, C, shows, for instance, recordings made from the roots of the 3rd and 5th ganglia when only the ipsilateral first root of the 4th segment remained intact. Even following the severance of the last remaining first root the rhythmicity persisted in several preparations. In most cases the greatest similarity recorded before and after cutting of this last root occurs in the period immediately following complete isolation. Later, intermittency may be recorded over a range of frequency of about 5–60 per minute (Text-figs. 2B, D; 3A). In assessing the significance of these observations it is important to remember the variability in the discharge frequency which occurs in the isolated abdominal preparation when all roots remain intact, and especially the fact that rhythmic bursts also become fewer in such preparations as time elapses. But it is equally clear that discharges following complete isolation do not show the same clear patterning of the individual discharges obtained when the first roots are intact. It seems improbable then, that these intermittent discharges recorded from the isolated nerve cord would be able to produce the finely controlled movements of the swimmerets in the complete absence of any peripheral inflow. On the other hand, they appear directly connected with the movements. In support of this is the fact that the second and third roots show a type of 'spontaneous' activity, which is quite different from that recorded in the first roots. The second roots typically have units



Text-fig. 2. *Procambarus clarkii.* Isolated abdomen preparation. Activity in first roots of the 4th (upper beams) and 5th ganglia following progressive isolation of the abdominal cord. Preparation showing different types of intermittent discharges. Cuts from long records. A. All nerve roots cut except the contralateral first root of the 4th ganglion. Bursts of about 75/min. from 4th ganglion and 25/min. from the 5th ganglion. B. Abdominal ganglia completely isolated. Two types of bursts from 4th ganglion. C. Ten minutes after isolation. Larger bursts become predominant in upper beam (40/min.) but brief bursts in lower beam. D. Twenty minutes after isolation. Prolonged bursts on upper beam have become less frequent (4–5/min.). Time marker: 1 sec.

which discharge continuously (Wiersma, 1952) sometimes with gradual changes in frequency but only very rarely intermittent firing, though there may be a tendency to form doublets (Text-fig. 3 B). This indicates that the intermittent bursts in the first roots are not due to anoxia or general deterioration of the preparation as a whole. There can thus be no doubt that within the cord there exists a mechanism capable of showing just the sort of intermittency which is found when all sensory mechanisms are intact. These central mechanisms exist for each of the swimmerets which, as mentioned earlier, are individually capable of independent activity unco-ordinated with their neighbours.

As to the nature of the mechanisms involved in the integration of the activity between different swimmerets, the isolated ganglion preparation is not so helpful. In some cases recordings from first roots of different segments have shown distinct phase relationships when only a single root remained intact, but there were few instances when this was clear in the completely isolated cord. More often the recordings of different roots showed independence from one another, but normal phase relationships were fairly common when the isolated cord was driven from above as is described in the following section.



Text-fig. 3. *Procambarus clarkii.* Isolated abdominal ganglia. A. First root activity from the 4th (upper beam) and 5th ganglia showing rapid intermittency (120/min.). B. Typical pattern of activity in second roots of the 5th ganglion. Time marker: 60 cyc./sec. and 1 sec.

(4) *The influence of 'higher' centres*

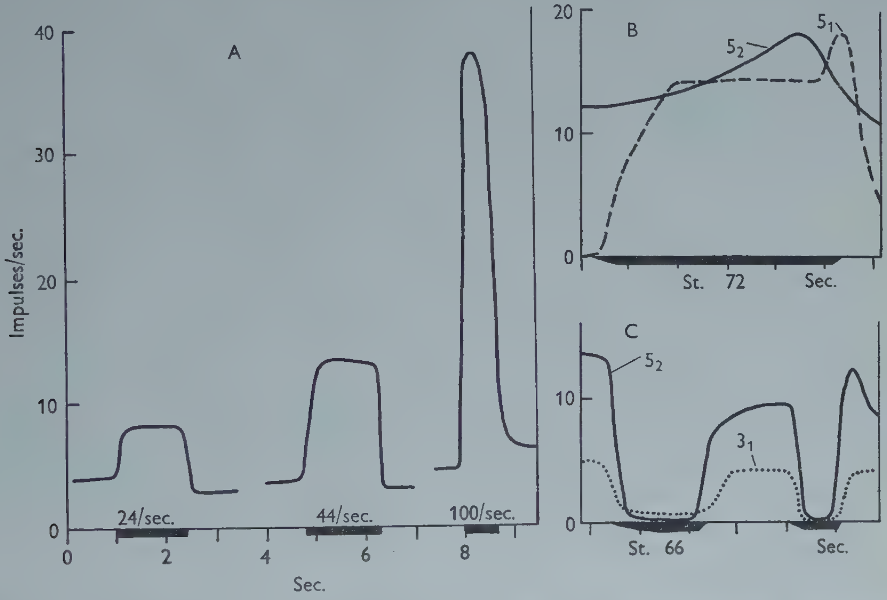
Movements of the swimmerets in the whole animal may be brought about by lifting the walking legs off the ground. Rotation of the animal about its longitudinal axis causes an asymmetry in the activity of the swimmerets and uropods. These reactions, which do not occur in the isolated abdomen, point to a control by the higher nervous centres as does the more persistent rhythmicity after cutting the cord between thorax and abdomen. Direct evidence for the existence of such influences has been obtained by stimulating small bundles of nerve fibres in one of the circumoesophageal commissures. Although effects on swimmerets, both excitatory and inhibitory, have been obtained from stimulation of several areas, only one of these was found in almost every preparation and has received special study. Its effects have been analysed with the aid of ciné films taken of the resulting movements and also by making records from first root discharges during the stimulation. Because of the similarity of the results in different preparations it seems likely, but not proven, that a single fibre is involved. This fibre is found in the ventral, slightly medial part of the commissure (area 70; Wiersma, 1958). When the small active bundle is stimulated at a frequency of about 50/sec. or higher, the swimmerets immediately move into the fully retracted position, the 5th swimmeret leading as it does in the next phase in which the swimmerets begin to beat rhythmically with a frequency of 30–60/min. At first these beats consist of only slight returns towards the protracted position, but they gradually become larger. During the stimulation the swimmerets never reach the fully protracted position as is normal in the typical movements. However, after stimulation ceases the beats frequently continue and then often cover the full range of motion. The positions of the exo- and endopodite are also rather different from the 'normal' beat, at least during the stimulation. When the swimmerets are first in the fully retracted position, the exopodite and endopodite separate maximally from each other and stay in this position during the movements. Monophasic recordings from first roots during stimulation show a prolonged burst of impulses during the time that the other swimmerets are retracted, which changes into rhythmic bursts when the swimmerets start their rhythmic movements (Pl. 15, fig. 2). Again after discontinuation of the stimulus well-marked intermittent bursts are often present. In two roots of the same side these bursts show the expected phase relationships according to the relative location of the roots. Furthermore, the intermittency is restricted to the first roots and is not shown by efferents in the second or third roots (Pl. 15, fig. 2A).

In the light of the results obtained by the progressive isolation of the abdominal cord it was of interest to follow the same procedure in preparations where driving of the swimmerets was obtained from stimulation of specific areas in the circumoesophageal commissure. In such an experiment it was found that the effect of stimulating area 70 did not change fundamentally when all roots in the abdomen were cut except for the first root innervating a single swimmeret. This swimmeret showed the same typical response as was present before the other roots were cut, and the electrical recordings from two first roots initially cut were also not altered by additional cuttings. The stimulation produced in them a continuous discharge which then became intermittent and persisted after the stimulus was discontinued. It was of considerable interest to find that this driving persisted even when the last remaining root was cut and the abdominal cord became completely isolated from its own peripheral inflow. The intermittent bursts are presumably equivalent to those associated with retraction, and it is of interest that the less intense protractor bursts frequently found before isolation (Pl. 15, fig. 2A) were very rare in isolated preparations. It is true of course that the C.N.S. was not completely isolated from the periphery because stimulation in the commissure also has minor influences on the other appendages, but they do not show rhythmic movements so that it is unlikely that the abdominal cord is subjected to any intermittent sensory input. It is concluded, therefore, that stimulation of these descending pathways with a constant frequency of impulses influences some central mechanisms within the abdominal cord to produce the intermittent discharge of impulses in the complete absence of any sensory feed-back from the appendages, which are normally influenced by these efferent discharges. As we have seen the tendency to produce intermittent discharges is already present in the abdominal nerve cord and the stimulation only seems to accentuate this property. The presence of typical rhythmic movements in the isolated abdomen also precludes the suboesophageal ganglion from being the primary pacemaker. An interesting feature found in several preparations was that the phase relationships of the swimmeret movements and the motor discharges in the cut roots can be the same during 'driving' of the discharges by stimulation of the oesophageal bundle as in the intact animal. Here, too, the 5th swimmeret has the lead, as shown in Pl. 15, fig. 2D, where bursts in the root of the 5th segment precede those of the 3rd segment. This is unexpected since, presumably, the anterior ganglia will be stimulated first by the descending pathways. Since cutting of the 5th ganglion does not stop the discharges in a more anterior ganglion (Pl. 15, fig. 2E), it is certain that the descending pathways do also have direct connexions with more anterior ganglia. This indicates that the reason for the leading function of the 5th ganglion is its lower threshold, or differently stated, its higher state of central excitation.

An illustration of the relative independence of the individual centres for swimmeret movements may also be seen in Pl. 15, fig. 2E, where rhythmicity is clearly present in the 3rd but not in the 4th ganglion. In this instance the cutting off of the 5th ganglion may be at least partially responsible, but similar observations have been made when the whole abdominal cord was still intact, though these are rare.

Variations in the frequency of stimulation produced differences in both the observed activity of the swimmerets and the nature of electrical activity in the first roots. At low frequencies (5–10/sec.) no movements were detectable, but a very slight increase in impulse discharge occurred in the first roots. Over a narrow range of frequency (*c.* 30/sec.) slight twitches could be seen in the swimmerets and evidence for direct driving of units in the first roots at the stimulation frequency was observed. Raising the frequency now produced the increased discharge in the roots associated with the tonic backwards movements of the swimmerets followed by intermittent bursts which persisted when stimulation was discontinued. The frequency of these bursts did not appear to be influenced by further increases in frequency, although the initial discharge was increased and the rate of the swimmeret retraction was more rapid.

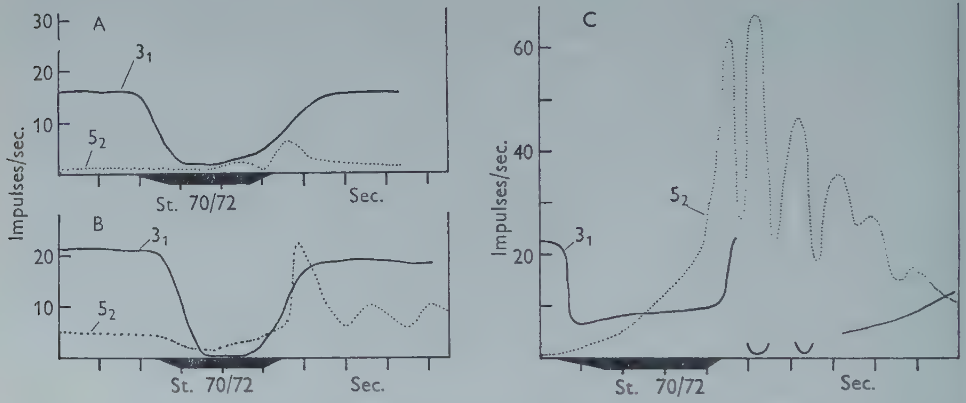
In marked contrast to the gradual development of the swimmeret beats on stimulation of the fibre from area 70, it was noted that continuous stimulation of a fibre in area 64 caused an immediate rhythmic beating quite like spontaneous metachronic waves. Probably because of the presence of an inhibitory interneurone in the immediate neighbourhood of this fibre, it has been prepared successfully in only very few preparations, and hence no definite statements can as yet be made about the root discharges and the order in which the swimmerets beat under different circumstances when this fibre is stimulated. In addition to these observations on the release of intermittent discharges in the first roots of the abdominal cord clear evidence has been found for the existence of inhibitory and excitatory effects on single units in the first roots by stimulation of other fibres or small bundles in the oesophageal commissures. For instance, Text-fig. 4A shows the excitatory effect on a tonically discharging unit when stimulation was applied to a small bundle in area 72/74 of the commissure. In this particular example the root was on the contralateral side but similar effects are found in ipsilateral roots also. It will be seen that the frequency of discharge is clearly related to that of the stimulus in this instance, but this is not always quite so clear. The excitatory effect of stimulating area 72 on a unit in an ipsilateral first root of the 5th ganglion is shown in Text-fig. 4B whereas another unit (5_2) is not so much affected. On the other hand, the latter unit is very strongly inhibited by stimulation of a bundle in area 66, as was another unit in the first root of the 3rd segment (Text-fig. 4C). Behaviour of single units in another preparation following stimulation of a bundle between areas 70 and 72 is shown in Text-fig. 5A, B, C. A unit in the 3rd segment shows very clear inhibition



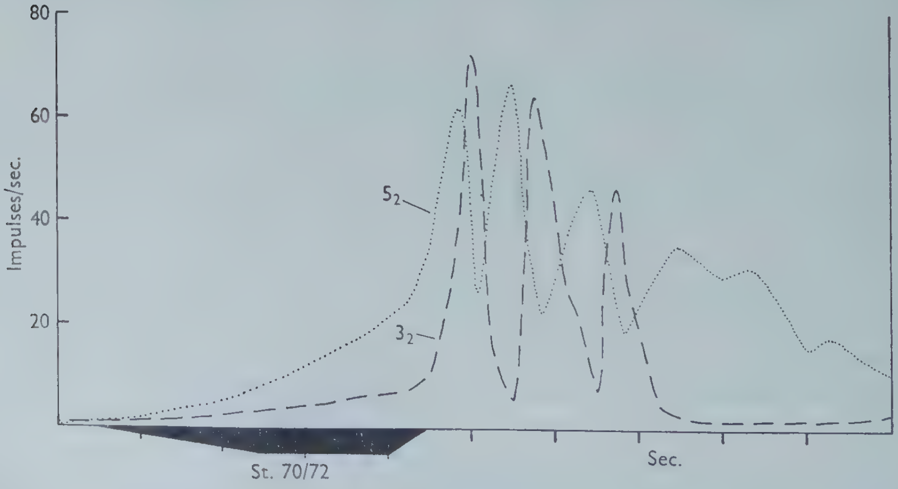
Text-fig. 4. *Procambarus clarkii*. Abdominal nerves intact except those from which recordings are shown. A. Effect of stimulating a small bundle of fibres in area 72/74 of the circumoesophageal commissure at different frequencies on the discharge of impulses in a single unit of the contralateral first root of the 3rd ganglion. B. Impulse frequency in two units of the ipsilateral first root of the 5th ganglion following stimulation (50/sec.) of a bundle in area 72. C. Same preparation as B, stimulation in area 66 produces inhibition of units in the first root of the 3rd and 5th ganglia.

whereas another unit in the same root (3_2, Text-fig. 6) and also one in the 5th segment shows a slight excitatory effect during the stimulus with a very pronounced increase in discharge frequency when the stimulation is stopped. In record C (Text-fig. 5) this resulted in rhythmic intermittent bursts in these units as the swimmerets were excited and then units in the two roots were out of phase (Text-fig. 6).

Although examples of these types of influence from the brain could be found in most preparations, it was difficult to repeat a particular response consistently in several preparations (with the exception of the effects on the swimmerets from area



Text-fig. 5. *Procambarus clarkii*. Abdominal nerves intact except those from which recordings are shown. Changes in frequency of impulses in two units (3_1 and 5_2) from the ipsilateral first roots of the 3rd and 5th ganglia produced by stimulation of a bundle of fibres in area 70/72 of the circumoesophageal commissure. The frequency of stimulation was 50/sec. in all cases and the intensity was raised gradually in each experiment, but maintained at a constant level for about 2 sec. The three graphs (A, B and C) show the effect of progressively increasing this final intensity.



Text-fig. 6. *Procambarus clarkii*. Same experiment as Text-fig. 5 C. Rhythmic changes in the frequency of single units in the first roots of the 3rd and 5th ganglia following stimulation (50/sec.) of a small bundle in area 70/72.

70 reported above). Moreover, variability was encountered in the effects of stimulating the same bundle in a given preparation. It was for this reason that the investigations were not carried further, but the results obtained undoubtedly give objective evidence for pathways by which the brain can exert the excitatory and inhibitory effects which have been ascribed to it on many occasions.

DISCUSSION

The observations described above are of interest in relation to the much-discussed problem of whether rhythmic movements are primarily due to occurrences in the C.N.S. or are triggered by peripheral sensory inflow. Rhythmic electrical activity in the deafferentated C.N.S. has been considered as favouring the first viewpoint (Adrian, 1931; von Holst, 1937). On the other hand, evidence has been obtained that in order to make co-ordinated limb movements possible, at least one limb must have both afferent and efferent innervation intact (Gray, 1950) and a similar finding was made with respect to the leech, in which rhythmic electrical discharges were present in the cord as long as a limited region of the body was connected to it, whereas after isolation of the cord no comparable rhythmicity obtained (Gray, Lissman & Pumphrey, 1938). Our results on the *isolated* abdomen are in fairly good agreement with this latter view, for the rhythmic discharges which can be led off from a preparation with only a single first root intact are essentially similar to those of the intact cord; but this is not true when this last remaining root is severed. It is thus evident that proprioceptive impulses are of importance for normal co-ordination of the swimmerets under these conditions. However, a certain rhythmicity can still be present in the spontaneous discharges even in the totally isolated cord. Since such discharges are absent from the other roots, which do not innervate the swimmerets, and since the discharges in the first roots, though greatly changed from those during regular beating, are still like those which can normally occur when the swimmerets are not well co-ordinated, it appears that they do represent abortive attempts at rhythmic swimmeret movements. This view is greatly strengthened by the fact that stimulation of certain central nerve fibres is capable of inducing quite regular and normal-looking rhythmic discharges in preparations where the abdominal cord is totally isolated from the periphery but connected to the higher part of the C.N.S. In this connexion it was of special interest to note that different central fibres exert different types of control, which are, however, independent of sensory inflow in the cord. Stimulation of the fibre in area 70, which was the most extensively investigated area, gives rise to a series of reactions, namely, first retraction and spreading of the rami and then gradually increasing movements of the swimmerets. It might have been expected that especially the second part of this sequence would depend on the local sensory inflow. However, this was obviously not the case, since after complete isolation the impulse outflow in the first roots retained its pattern of firing, uninterruptedly at first and in more and more pronounced bursts later. Though we succeeded only very few times in obtaining the fibre from area 64, the effect of its stimulation was also independent of the presence of local sensory inflow.

From these results we are inclined to conclude that, though sensory inflow from the swimmerets plays an important role in locally elicited rhythmic movements, it is without much importance for centrally driven rhythms. Whether or not the latter are to be considered as reacting to sensory stimuli, for example, from the head or thorax, or may fire 'spontaneously' is unknown. In this instance there can thus be no sharp distinction between a totally spontaneous action and one dependent on sensory input. But it is certain that the same typical metachronal movements of the swimmerets can be elicited in different ways, namely, by a local co-ordinating mechanism in the cord, in which sensory input plays an important part, and by specific 'regulatory' nerve fibres from the brain, for which no other sensory input is necessary. In addition the brain can, by a similar mechanism, inhibit the swimmeret movements. The whole mechanism of the nervous control of the swimmeret movement reminds one strongly of that in the neurogenic heart ganglion of the decapods (Wiersma & Novitski, 1942; Maynard, 1955; Bullock & Terzuolo, 1957). Whereas in the heart ganglion single cells may show individual spontaneity, as they do in the centres of each swimmeret, there is in the abdominal cord, in addition, a co-ordination between the different ganglionic centres, which is still weaker. Even during central driving it was noticed that not all swimmerets need to give the same number of beats, though this is more often the case than when the cord is isolated.

As a hypothesis, the following picture may be proposed. (*a*) Each local ganglion contains a centre of co-ordination for each swimmeret. Local sensory inflow will tend to impress a certain pattern of rhythmicity into the firing order and firing frequency of the different motor neurones, which leads to a full swimmeret 'stroke'. However, any variation can occur, related to the 'activity state' of the centre. (*b*) Interconnexions between the different centres exist, which show a delay time accounting for the tendency of anterior swimmerets to beat later than posterior ones. It is likely that usually the last pair of swimmerets (normally the 5th) has the strongest influence in this respect. It is uncertain whether similar connexions in a posterior direction are present, though there are reflex connexions in this direction (this was shown by the fact that pushing the 2nd swimmeret backward may trigger swimming movements, especially on the ipsilateral side). (*c*) There are interneurones coming from the brain, which by continuously firing impulses can trigger the centres of the swimmerets to rhythmic firing. This may be based on the increased 'activity state' which they bring about. (*d*) Conversely there are interneurones which stop swimmeret movements, presumably by decreasing the activity state.

In view of the fact that the single heart ganglion does not readily yield to a full analysis of the underlying events (Bullock & Terzuolo, 1957) it is not likely that the many mutual influences which are present in the abdominal cord can be completely analyzed. But the 'release' of a motor pattern through the stimulation of central interneurones is strongly indicated as the most likely explanation for swimmeret movements. This release can also be brought about by sensory input of local nature.

SUMMARY

1. Electrical activity recorded in the first roots of the abdominal nerve cord show bursts of activity which accompany rhythmic movements of the swimmerets. These bursts persist when a root is cut distal to the electrodes.

2. Proprioceptive impulses have been recorded from the distal portion of these nerves during passive movements of the swimmerets. They have also been recorded in the connectives during both passive and active movements.

3. Cutting all second and third roots of the abdominal ganglia does not interfere with the rhythmic swimmeret movements of the isolated abdomen. Rhythmic efferent bursts persist in first roots even when only a single first root remains intact in such a preparation.

4. Intermittent bursts are also found in the first roots of a completely isolated cord but their pattern, frequency and phase relationships differ from those recorded in the same roots before isolation was completed. Such bursts are very rare in second and third roots.

5. Stimulation of small bundles of fibres in the circumoesophageal commissure produces well-defined inhibitory and excitatory effects on the discharge of single units in the first roots.

6. One specific bundle found in many preparations, when stimulated at 50/sec., caused a tonic retraction of all the swimmerets which then began to beat rhythmically and continued to do so when stimulation was discontinued.

7. Intermittent bursts in the first roots took place with correct phasing during this stimulation even when the abdominal ganglia were isolated except for their connexion with the last thoracic ganglion. Again, such bursts were absent in the second and third roots.

8. It is concluded that both the inflow from peripheral proprioceptors and intrinsic properties of the central ganglia play essential parts in the co-ordination of the metachronal movements of the swimmerets.

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EXPLANATION OF PLATES

PLATE 15

Fig. 1. *Procambarus clarkii.* Isolated abdomen preparation. Electrical recordings from small bundles of fibres in the right connective between the 3rd and 4th ganglia. For position of areas see Wiersma & Hughes (1961). A. Area 76. Discharges particularly associated with retractor movements of the 4th and 5th pairs of swimmerets. Tonic discharge occurs in the same units when these swimmerets were in a partially retracted position. Cutting of this bundle produced retraction particularly in the ipsilateral 4th swimmeret. B. Area 76. Similar record from another preparation. C. Area 80. Bursts not apparently associated with any rhythmic swimmeret movements. Time marker: 60 cyc./sec. and 1 sec.

Fig. 2. *Procambarus clarkii.* Recordings of efferent impulses in abdominal roots during and following stimulation (about 50/sec., indicated by arrows) of small bundles in the circum-oesophageal commissure. A. Intact preparation showing difference between first root (upper beam) and second root activity following stimulation of area 72. B. Intact preparation; activity in first roots of 3rd (upper beam) and 5th ganglia. Stimulation of area 70 in the ipsilateral commissure. C. Faster recording of intermittent bursts in first roots of the 3rd and 5th ganglia of an intact preparation. Stimulation of area 70/72. D. Same preparation as B but all segmental nerves of abdominal ganglia 1–5 severed and connectives between 5th and 6th ganglia cut. E. Same preparation as B and D. 5th ganglion now removed. Recordings from first roots of 3rd (upper beam) and 4th ganglia.

PLATE 16

Procambarus clarkii. Isolated abdomen preparation. All second and third roots and connectives between 5th and 6th ganglia severed. Recordings of activity in first roots of the 3rd (upper beams) and 5th ganglia cut distal to the electrodes. Cuts from long records. A. All first roots intact except those from which recordings are made. B. All first roots cut except those of the 4th ganglion. C. Only ipsilateral first root of 4th ganglion intact. D. Same preparation as B but all segmental nerves of abdominal ganglia 1–5 severed and connectives between 5th and 6th ganglia cut. E. Same preparation as B and D. 5th ganglion now removed. Recordings from first roots of 3rd (upper beam) and 4th ganglia.

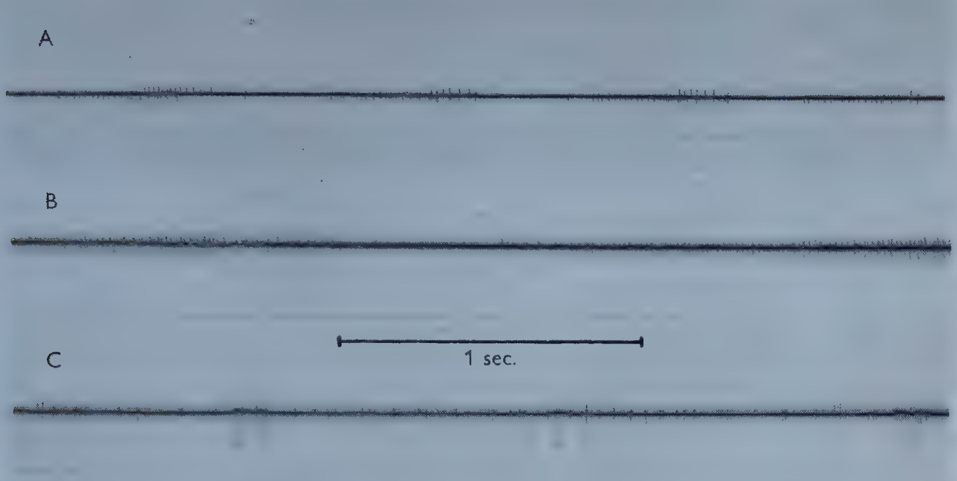

Fig. 1

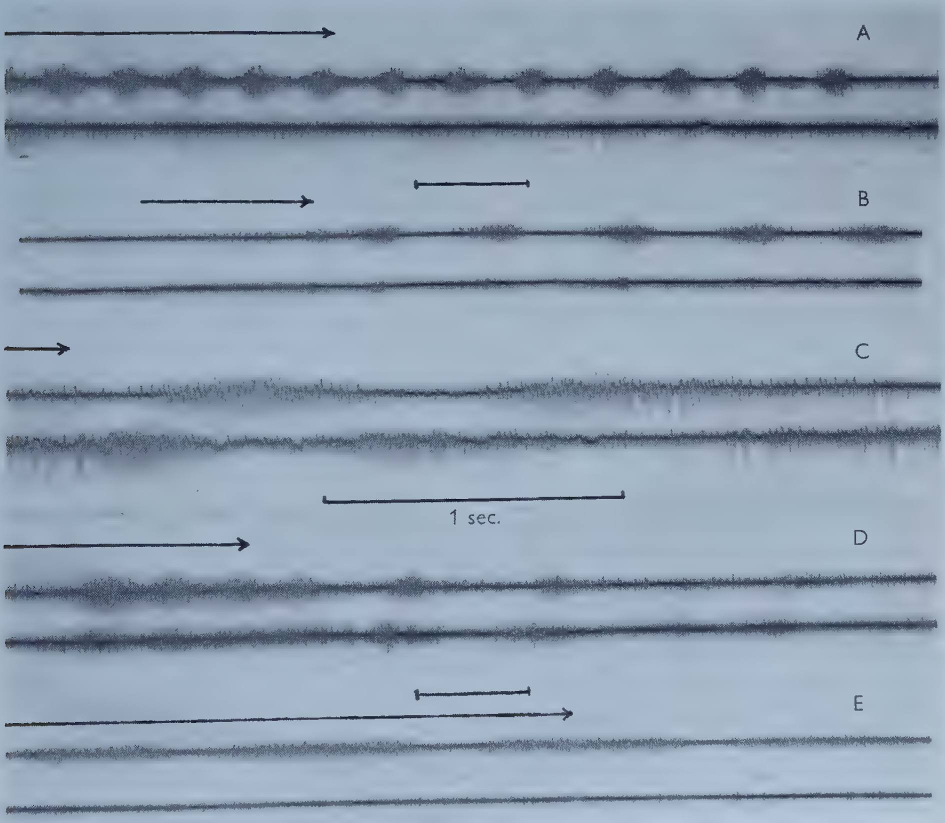

Fig. 2

HUGHES AND WIERSMA—THE CO-ORDINATION OF SWIMMERET MOVEMENTS IN THE CRAYFISH, *PROCAMBARUS CLARKII* (GIRARD)

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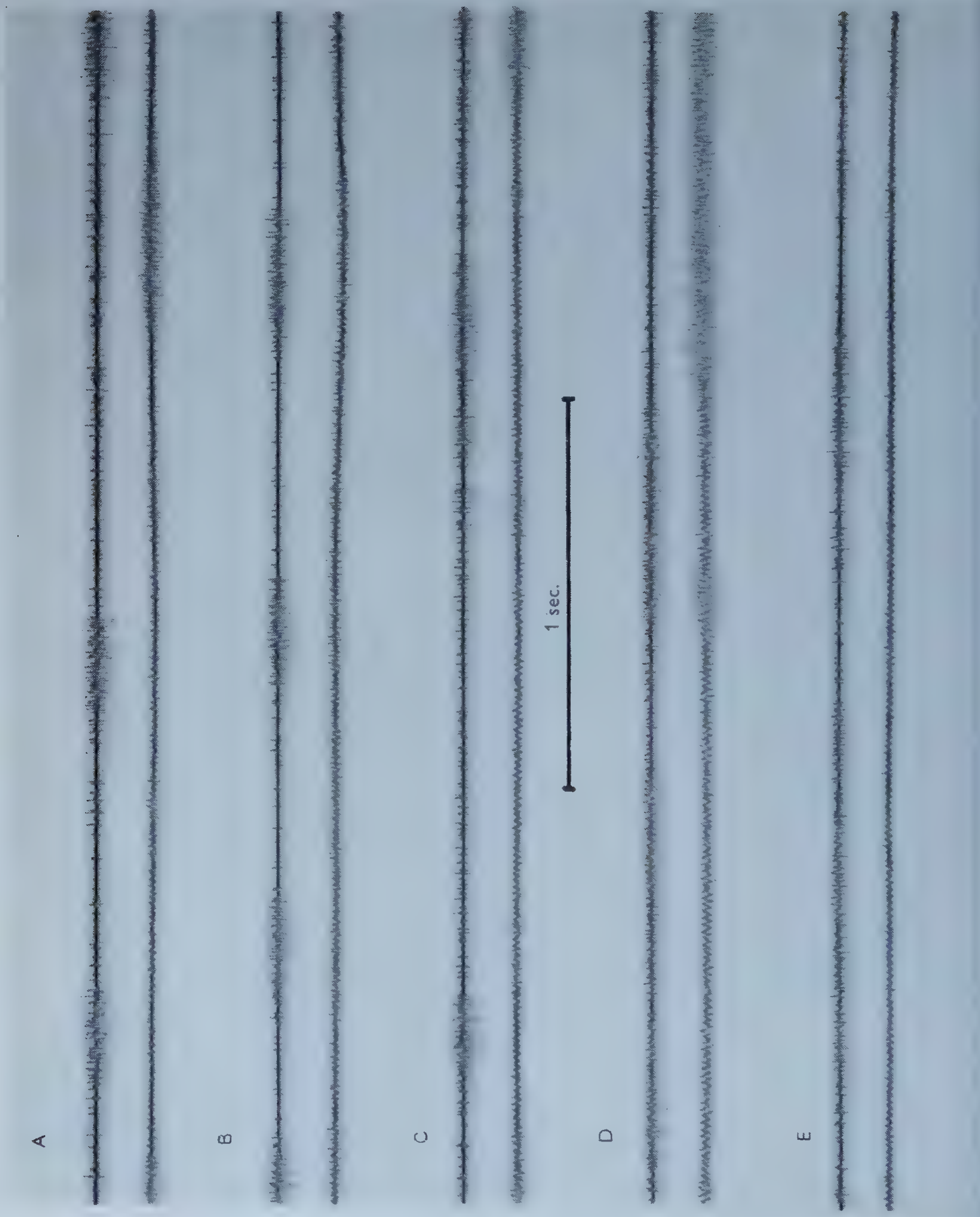

HUGHES AND WIERSMA—THE CO-ORDINATION OF SWIMMERET MOVEMENTS IN THE CRAYFISH, *PROCAMBARUS CLARKII* (GIRARD)

NEUROMUSCULAR ACTIVITY IN THE SWIMMING SEA ANEMONE, *STOMPHIA COCCINEA* (MÜLLER)*

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INTRODUCTION

The spontaneous activity and responses to electric stimulation of actinians have been intensively studied (e.g. Pantin, 1935*a*, *b*, *c*, *d*; Ross, 1952, 1957*a*, *b*; Horridge, 1958) for the light which they might throw on the functioning of the 'elementary nervous system', i.e. a nervous network lacking the physical concentration of neurones found in ganglia and brains. It seems desirable to add to this knowledge data from the swimming sea anemone *Stomphia coccinea*. This animal, although not yet well known, is evidently widely distributed, having been recorded from Millport, in the Clyde, Scotland (Stephenson, 1935), and from President Channel, Puget Sound, Washington, U.S.A. (Yentsch & Pierce, 1955). It should be pointed out, however, that the species identity of animals from the two regions has yet to be established with certainty. Stephenson (1935) noted that *Stomphia* is capable of becoming curiously active (for a sea anemone) twitching its 'tail' and 'jumping'. Similar activities can be elicited from the Puget Sound specimens by two species of starfish from the same region (although not the same habitat), *Dermasterias imbricata* and *Hippasteria spirosa* (Yentsch & Pierce, 1955; Sund, 1958).

Details of the swimming activity have been described by Sund (1958). Since the species reacts to the starfish named, by giving a set of distinct reactions which are usually executed in the same sequence, it can be said to show a high level of integrated activity, possibly higher than any known in other actinians. The following distinct parts of the response can usually be observed, in the following sequence. The division and resulting emphasis is based on my own observations, but closely follows Sund's description:

(1) Elongation, with uptake of water.

(2) Increase in turgidity with expansion of oral disk.

(3) Waving of whole oral disk, usually in a circular motion.

(4) Release from substratum by extrusion of centre of basal disk and withdrawal of edges.

(5) Alternate and rotating sequence of quick flexions causing 'swimming'.

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* Aided by contract (NR 104-142) between the Office of Naval Research, Department of the Navy and the University of Washington.

In addition to these complex reactions, there are almost certainly other, slower, forms of behaviour, which might be equally complex and meaningful. The reactions of the animals to starfish were analysed closely by Sund (1958) and by Ward (1958), who have shown that the elicitation of swimming depends upon a chemical substance exuded by the starfish. Sund found that swimming can also be elicited by electric shocks, and this has been further studied by Wilson (1959). The nature of responses to electric stimulation has not, otherwise, been investigated. Sund found that a single stimulus was not followed by a response, but that a contraction occurred in response to a second shock delivered 1 sec. after the first. He does not give sufficient details to decide exactly which muscles were responding. Wilson (1959) also found responses occurring only to the second shock and subsequent, closely spaced stimuli. He found that in the eighteen animals he examined, most of them gave swimming responses, comparable to those obtained by presenting *Dermasterias*, when *precisely* eight electric shocks were applied to the base of the body wall. This quite remarkable finding demands further investigation. The total number of shocks was much more important than other parameters such as stimulus strength (no effect), duration (no effect) and in particular the interval (frequency) which could be varied between 0·25 and 2 sec. without affecting the response. Sund (1958), who had also given eight shocks as the approximate number required to evoke swimming, still obtained the response at intervals of only 0·12 sec., with a total of eight shocks. Wilson states that at the higher frequencies (above four per sec.) more than eight shocks are required and that at intervals longer than 3 sec. the response does not occur.

Wilson also studied the effect of applying what he called the 'subthreshold' number of seven stimuli, which he repeated at intervals. He found that seven stimuli became adequate to evoke swimming after a single (unsuccessful) trial of seven. The initial stimulus he referred to as 'conditioning' and the subsequent ones as 'test' stimuli. The latter could be applied as long as 1 week after the first and still be effective. Wilson proposed to call this phenomenon 'long-term facilitation'. Subsequent to the 'conditioning' experiment, in which an animal came to respond to seven shocks, it could now be made to respond to six shocks by the same procedure, namely a single application of six shocks.

Some of the animals responded to a single contact with *Dermasterias*, others did not. The latter would, however, react to a smaller number of electric stimuli than usual (i.e. five, six or seven instead of the usual eight) by swimming if the shocks were applied soon after the starfish contact. Once the anemones had then reacted to this mixed stimulation they always subsequently responded to a single contact with the starfish. Wilson argued that there had been a long-lasting decrease in 'threshold' of the response. In looking for a site of the change he argued that since unlike stimuli (chemical, from the starfish, and electrical) effectively summated, the myoneural junction might be the site of a facilitation process giving the over-all appearance of a lowering of 'threshold' (= no. of shocks required to elicit) of the complex swimming response.

It is difficult to see the logic in this argument. Does it mean that the smaller number of stimuli which cause swimming after 'conditioning' were eliciting the response from the nervous system all along but weakly so that the muscles did not respond? Why should an additional stimulus increase their intensity? Or does it fire a trigger mechanism? How could such a long-lasting 'facilitation' come about physiologically anyway? Although such questions cannot be answered, the major premiss, namely that there is a long-lasting facilitation at the level of the neuromuscular junction, can readily be tested provided an effective nerve-muscle preparation can be made of the responding muscles. The present work was undertaken principally with a view to finding out if the state of 'facilitation' in the excitation/response pathways using electric stimuli could be shown to undergo 'long-term facilitation'. At the same time a number of other observations were made on the animals, and these are reported here. It was not found possible to confirm the observations of Wilson, and many observations contrary to his views were made.

Animals were allowed to remain on their host shells (*Modiolus modiolus*, a mussel) or else they were induced to swim away by touching them with *Dermasterias* and then made to settle on paraffin wax in glass dishes. Once settled, they were pinned to the wax by a number of small entomological pins pushed through the margin of the basal disk. Animals prepared in this way would live quite well for up to 2 weeks, after which they were released. For recording, hooks made from entomological pins were passed through the body wall under the oral sphincter muscle and attached by linen threads to lightly weighted isotonic levers. They were kept in aerated, fresh sea water which was changed every few hours. Electric stimuli were applied through glass tubes with sea water applied to the surface of the animal, and containing thick chloride-coated silver wire. The stimuli were direct current pulses derived directly from Grass S 4 electronic stimulators and delivered at constant voltage. The stimulus duration was usually 0·5 msec., or from 0·1 msec. up to a maximum of 5 msec. The temperature of the water rose gradually from about 12° C. (slightly more on hot days) to 16° C., when it was changed. Animals of various sizes were tested, the smallest being 2 cm. in diameter at the basal disk. The largest one available had a disk diameter of about 8 cm. at the time of testing, and its length reached 10 cm.

RESULTS

SPONTANEOUS ACTIVITY

Time-lapse films of *Stomphia* made by Sund and Dr Dixie-Lee Ray show that the anemones are incessantly active, extending, contracting, bending, swaying, swirling, rippling and twisting in a great variety of motions, all of which are executed very slowly. Previous experience with other animals exhibiting 'spontaneous' activity (ascidians—Hoyle, 1952) had made me appreciate the need for taking into account the fluctuating states of an animal if one is to obtain consistent results when studying stimulus/response patterns in the whole animal. Accordingly, recordings were made of the spontaneous activity patterns of most of the animals which were tested electrically.

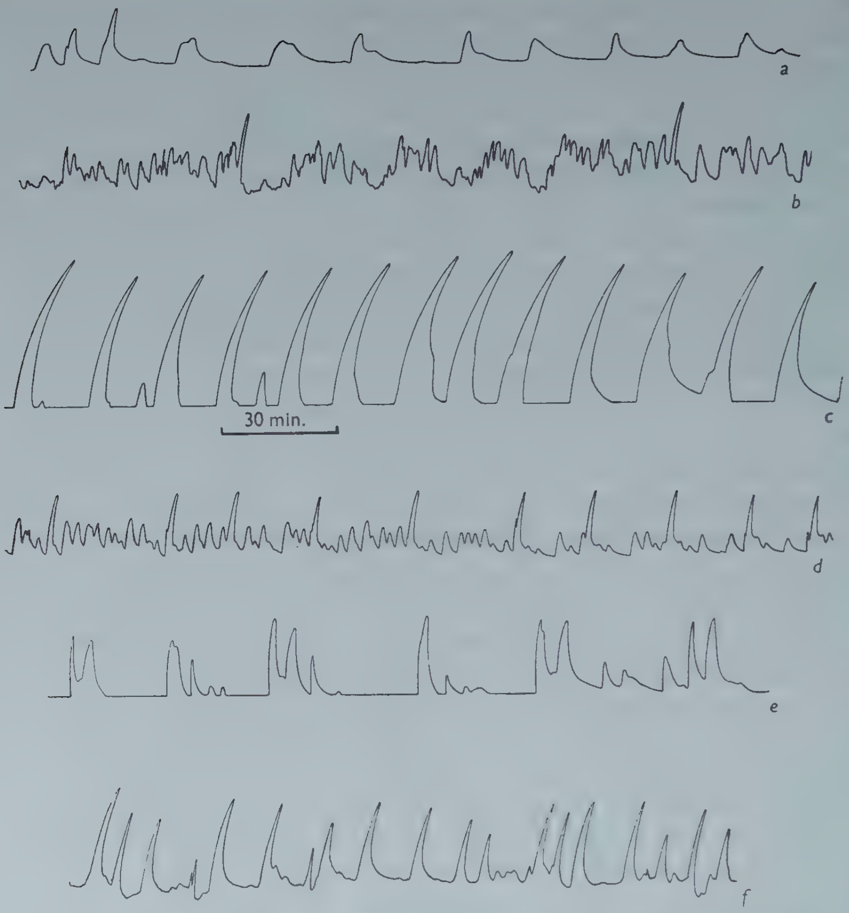

Fig. 1. Examples of spontaneous activity recorded from six different specimens of *Stomphia coccinea*.

In the tanks in which the *Stomphia* were kept, in running freshly-pumped sea water, it was evident that several of the specimens tended to be slightly shrunken during the day and expanded at night. There was, however, little evidence of a marked nocturnal/diurnal rhythm such as was recorded by Batham & Pantin (1950) in *Metridium* in the kymograph records of *Stomphia*. The specimens were equally active both during the day and at night, with only a slight extension occurring in the dark (i.e. at night). They were not given any food during the testing period. A rather bewildering variety of records was obtained, some examples of which are illustrated in Figs. 1 and 2. Some specimens gave an irregular pattern of slow movement, not involving any powerful contractions. Others gave, at fairly regular intervals, slow complete retractions always followed by a gradual extension to the fully expanded position. In the intervals between large contractions there were smaller movements due partly to bending, partly to contractions of the circular muscles.

One feature which was observed more than once, in different animals, was a full retraction followed by full extension and then a very gradual shortening caused by relaxation of the circular muscles, the whole process taking about half an hour. This movement would be repeated after about 2 hr. and again after a further 2–3 hr. (Fig. 2). An attempt was made to locate the origin of the spontaneous activity. Animals were anaesthetized in magnesium chloride solution and cut into rings; the anterior body wall including the oral sphincter and tentacles was prepared, and also rings from the basal region. These were allowed to relax in running sea water and then attached to kymograph levers. It was found that rings from both anterior and posterior regions showed marked periodic activity (Fig. 3). That from the tentacles and sphincter region was never very regular, and contained a number of small movements as well as larger, very slow, contractions. By contrast, the activity of basal rings was sometimes extremely regular, tending to repeat itself at intervals of about half an hour (Fig. 3*c*). This activity, although probably due to circular muscles, is strikingly similar to that observed in one of the whole animals, in which it was due to the parietal muscles.

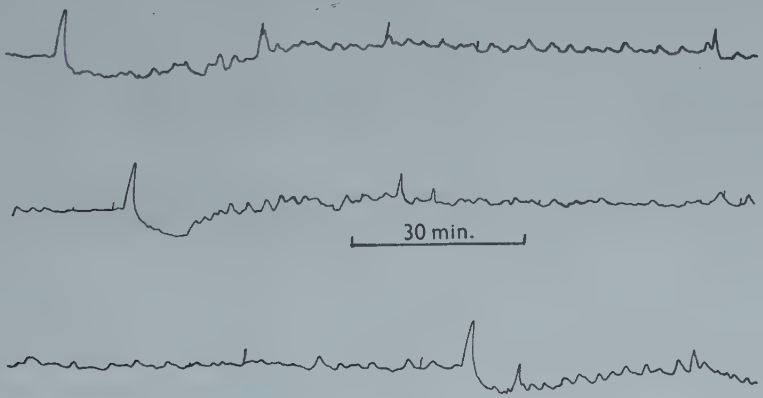

Fig. 2. Repeating pattern of slow activity of a whole animal not accompanied by strong periodic contractions.

These observations show that pacemakers capable of initiating muscular contraction are probably present in all parts of the animal. A pacemaker with a period of discharge of about half an hour is located in the basal region of the body wall.

These results recall those of Batham & Pantin (1954) obtained by recording from rings of body-wall of *Metridium*. In their experiments they went further than in the present ones, recording at the same time from four rings cut in the sphincter, subsphincter, mid-column and pedal regions, having a strip of body wall remaining to connect them together. Spontaneous, recurring slow contractions were found in all rings, particularly the pedal. There was only a very limited co-ordination of the contractions, showing the presence of pacemakers in each ring.

If any generalization can be made from these observations it is that each animal behaves differently from all others in detail, but that there is a marked tendency in all of them for certain movements to be repeated at fairly regular intervals, i.e. there is an inherent rhythmicity of action, comparable to and sometimes much more regular than that found in *Metridium*.

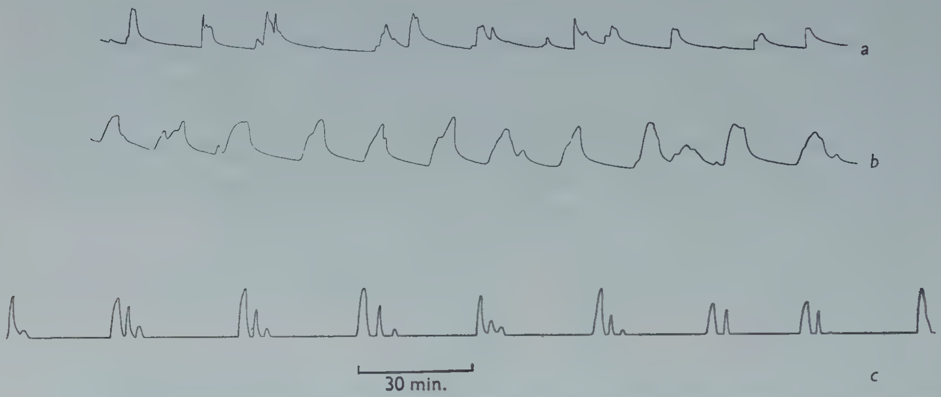

Fig 3. Spontaneous activity of isolated rings of body wall. *a*, Oral disk region; *b*, *c*, basal disk region. *a* and *b* were from the same animal. Note the very regular periodicity in *c*.

Responses to electric shocks

All the major muscle fields (Batham & Pantin's usage, 1951) found in sea anemones are well represented in *Stomphia*, and its parieto-basilar muscles are much more powerful than those of most others. They are the muscles responsible for the 'swimming' action. Since the animal is a fluid-filled sac, contractions in any of the muscles may be expected to produce a distortion which will be registered by the recording levers. Hence it is particularly desirable to confine the stimulation so that only one type of muscle responds.

The muscles present are: longitudinal retractors, parietals, parieto-basilars, circular muscles of the column, radial muscles and the oral sphincter. A diagram of their distribution is given in Fig. 4. The distinct oral sphincter can be isolated and studied independently, as was done by Ross (1957*a*) in *Calliactis*, but the responses of the other muscles (except the radial) can be studied only indirectly, in the whole animal, in which case it is impossible to be certain that only one type is contributing to the record. Nevertheless, a fairly complete effective separation is possible.

To obtain results of reasonable consistency from any muscle in the intact animal, the tests should be made only during the periods of relative quiescence which occur between major retractions. Even then, very considerable variations are encountered in threshold, magnitude of response and rate of relaxation.

(1). *Parieto-basilar muscles*. The reactions of these muscles cannot be distinguished with any degree of certainty from those of the parietals, but in other sea anemones in which the probable response of the parietal muscles is known it consists only of a slow contraction followed by a very slow relaxation. The magnitude is increased by increasing the number of stimuli applied, but the rate of rise is not greatly affected. In *Stomphia* the parieto-basilars are well developed and there is a very quick, powerful contraction of the parietal group which is probably exclusively determined by the parieto-basilars. This response can therefore be separated from that which the two sets of muscles might show in common, which is a much slower contraction resembling that attributed to the parietal muscles in other sea anemones. There is at present no evidence to decide whether the slow response is the exclusive product of the parietal group, or whether it also involves the parieto-basilars, which would then be dually responsive.

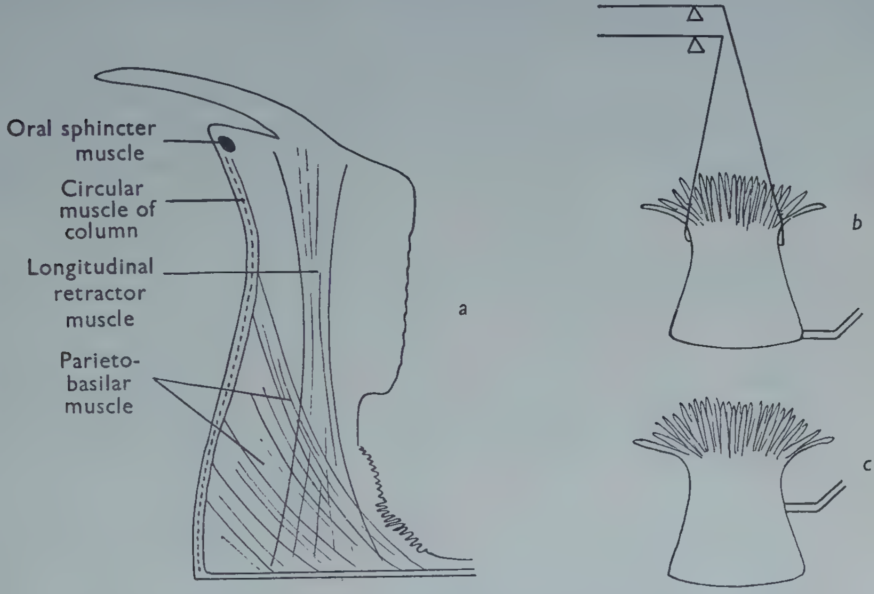

Fig. 4. *a*, Diagram of the principal musculature of *Stomphia* as seen in a perfect mesentery. The parieto-basilar muscles are strongly developed and attached to points high up on the column. *b*, Position of stimulating electrodes for eliciting responses of parieto-basilar muscles. *c*, Position of stimulating electrodes for eliciting responses of circular muscles of the column or of the longitudinal retractors.

A possible comparable quick response has been observed in the South American anemone *Bunodactis*. When this animal is touched lightly on the column a quick contraction occurs on the touched side only (Pantin & Vianna Dias, 1952) which could be due to the parieto-basilar muscles.

Selective fast responses of the parieto-basilars could be obtained by placing the electrodes at the very base of the column. The response obtained, in the fresh animal, is characterized by a contraction occurring locally, in the region under the stimulating electrodes, which will be referred to as ipsilateral (Fig. 5). The contralateral side, if it is affected at all, is either induced to relax slightly (see below) or else it gives a slight movement due to the passive elastic effect of the downwards movement on the other side. If the stimulation is continued, the responding region extends round the animal until the whole set of parieto-basilar muscles tends to contract, to roughly the same extent, with each stimulus (Fig. 13).

The magnitude of the response to a threshold or any constant-strength stimulus is very variable. It may vary in an irregular way, or it may wax and wane with a slow rhythm, if the stimulus is repeated at regular intervals of say 1 per 10 sec. (cf. Fig. 13). Responses may fail to occur for a time, only to return suddenly or gradually. The existence of this great variability makes it seem unlikely that the contractions are due to a direct excitation of the muscle fibres. Furthermore, there is no evidence of a direct excitation of the circular muscles, which lie between the electrodes and the parieto-basilars. Instead, it seems probable that the muscles are excited indirectly, via the local nerve net, the elicited excitation summating with the background activity occurring there.

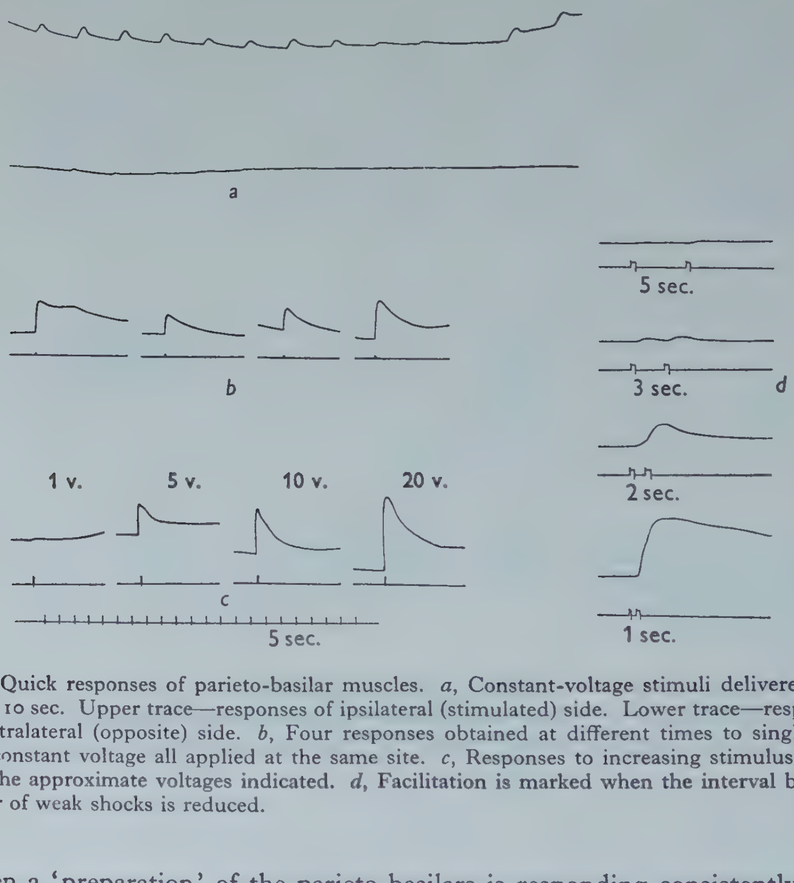

Fig. 5. Quick responses of parieto-basilar muscles. *a*, Constant-voltage stimuli delivered at one per 10 sec. Upper trace—responses of ipsilateral (stimulated) side. Lower trace—responses of contralateral (opposite) side. *b*, Four responses obtained at different times to single shocks at constant voltage all applied at the same site. *c*, Responses to increasing stimulus strength at the approximate voltages indicated. *d*, Facilitation is marked when the interval between a pair of weak shocks is reduced.

When a 'preparation' of the parieto-basilars is responding consistently, it can be shown that the magnitude of the contraction increases with increasing intensity of stimulation (Fig. 5*c*). This may in part be due to an increase in the area contracting, i.e. in the number of mesenteries brought into action. It appears visually also to involve a greater extent of local contraction, but this cannot be stated with certainty. Very strong stimuli appear to cause a small amount of active contraction also, on the contralateral side.

The fastest time required to reach peak contraction of a powerful parieto-basilar response in the intact animal is 0·2 sec. and relaxation takes 2–3 sec., although both times may be up to six times longer.

Possible reciprocal relaxation

The quick contractions of the parieto-basilars, when used in swimming, recur at intervals of only a few seconds, on opposite sides of the body. There is no doubt that a mechanism of reciprocal relaxation of the parietal muscles on opposite sides would be of assistance in ensuring rapid motion. Sund (1958) mentions the possibility of relaxation of the circular muscles on the same side, which would facilitate the ipsilateral bending. In many of the records obtained, both during swimming evoked by starfish and during electrical stimulation of the parieto-basilars, there occur small but sharply defined relaxations of the contralateral side which are almost synchronous with the contractions (Fig. 6). They might have been due, in some cases, to simple mechanical stretch consequent upon the increased hydrostatic pressure, but they were also visible when the mouth was open and when a glass tube was inserted through the mouth into the gastric cavity. Although by no means conclusive, these results suggest that a mechanism exists whereby the 'tonic' nerve discharge to the 'contralateral' parieto-basilar muscles is inhibited when the 'ipsilateral' ones are excited strongly. The inhibitory process is not likely to be at the level of the neuromuscular junction, because the ipsilateral side can be electrically excited by locally applied electrodes during the relaxation.

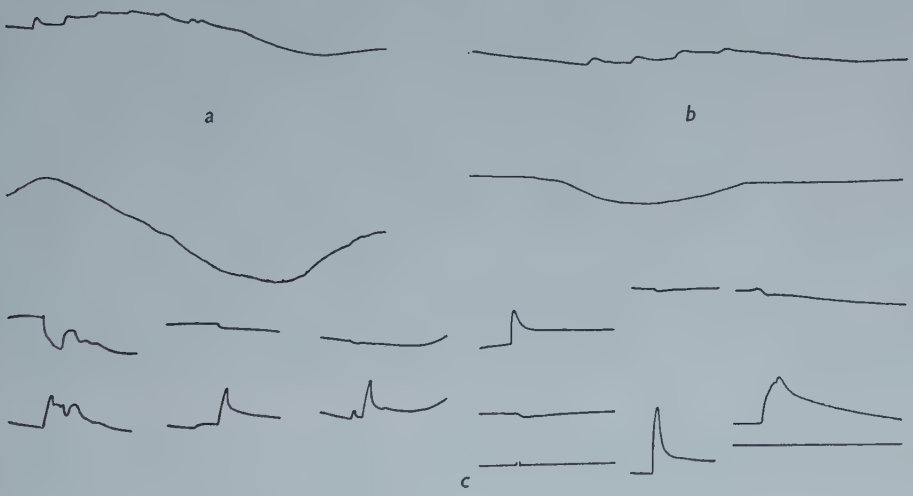

Fig. 6. Reciprocal relaxation in parieto-basilar muscle of non-stimulated side. Large contractions at side of stimulating electrodes. *a*, *b*, Short series of shocks applied at one per 10 sec ; *c*, miscellaneous responses to single shocks.

Summation and facilitation

Contractions of the parieto-basilars in response to successive excitations summate completely. If the first response was a large contraction, the second and subsequent ones are all much smaller and there is no facilitation. But if the initial contraction was small in magnitude due to a weak shock the response to the second shock is always much larger, showing a facilitation effect. In the less vigorous animals there may even be no response to a single shock, but the second or third gives rise to one. When the interval between two shocks is progressively reduced, a marked growth in facilitation is apparent (Fig. 5*d*).

(2) *Parietal muscles.* Following the application of a train of six or more stimuli through electrodes placed to obtain the parieto-basilar response, there occurs, as the animal relaxes after the quick parieto-basilar contractions, a strong, delayed contraction (Fig. 7). It is similar to that obtained by Pantin (1935*d*) in *Calliactis* and can be attributed to the parietal muscles. It does not occur at frequencies above two per sec., or below about one per 5 sec., being optimal at one per 2 sec.

(3) *Circular muscles of the column.* The best site to place the electrodes in order to obtain responses of the ordinary circular muscles of the column is about one-third of the way down the column, i.e. near the upper attachment of the parieto-basilars. Below this point one is liable to obtain responses of the latter. In this position two or more shocks delivered at intervals of 5 sec. elicit a slow contraction (Fig. 8), starting under the electrode but appearing to affect the whole circumference synchronously. The local contraction may spread down the column causing an appreciable elongation of the animal. There are also occasional small quick responses to the single shocks, and also delayed parieto-basilar 'incipient swimming' responses may appear. Although there may be no direct responses of the parieto-basilars, the nerve supply to the latter is certainly affected by stimulation at the point for optimal response of circular muscles alone.

(4) *Longitudinal retractor muscles.* These muscles can be excited with the electrodes in the same position as for exciting the circular muscles (3) (Fig. 8) and also at most other places on the column. The advantage of using the former position is that it is relatively free from interference by contractions of the parieto-basilar muscles. Responses of the retractors can be recognized by the way in which they all contract in synchrony, causing a sharp withdrawal of the marginal disk. Responses occur occasionally to single shocks. Laws & Ward (1959) found such responses in 17% of the tests they made, although it is not certain that they distinguished the responses from possible parieto-basilar ones. However, there is no doubt that the frequency of occurrence of responses of the longitudinal retractors of *Stomphia* to single shocks is several times higher than the figure of 0·3% found by Ross (1952) in *Metridium.* As in the latter the characteristic quick withdrawal response occurs to the second shock in a closely spaced pair, the amplitude increasing with decreasing interval between shocks. The response is reproducible, but with less consistency in regard to amplitude, than is the case in *Metridium.*

(5) *Oral sphincter.* The oral sphincter was prepared as an isolated structure after

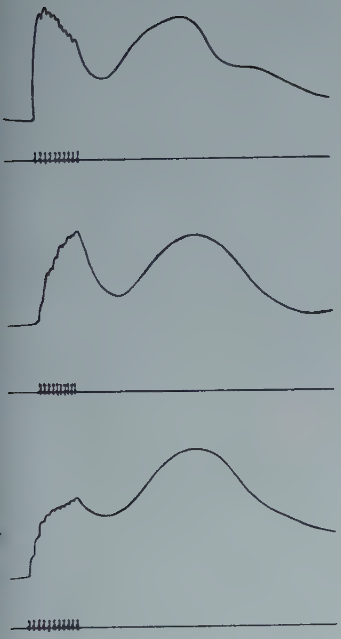

Fig. 7

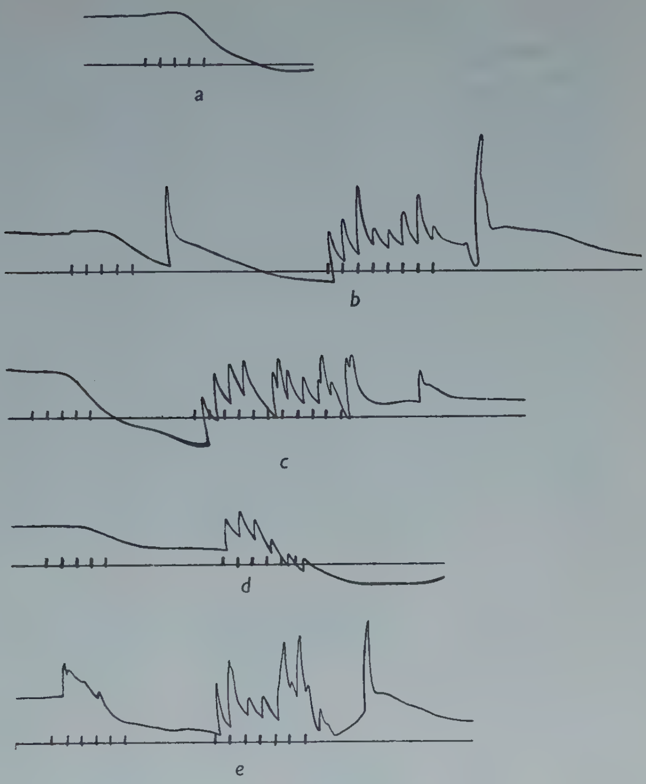

Fig. 8

Fig. 7. Slow responses of parietal muscles. Ten shocks were delivered at one per sec. There are initially summating quick responses due to the parieto-basilars and these are followed by the (probable) slow response of the parietal muscles. Note that in no case was swimming evoked by the shocks.

Fig. 8. Responses of circular muscles of the column and the longitudinal retractor muscles. *a*, Elongation due to local contraction of a ring of circular muscle under the stimulating electrodes evoked by single shocks at one per 5 sec. *b–e*, Various examples showing: first, reaction of circular muscles to single shocks, then reactions to double shocks at the same frequency (two per 5 sec.). The evoked quick contractions are due to the longitudinal retractors. Additional (spontaneous) contractions are due to parieto-basilar muscles. Note the quick relaxation in *b* as the contralateral side contracted first in incipient swimming. Contraction of the circular muscles was inhibited when the longitudinal retractors were activated in *b*, *c* and *e* but not in *d*.

first anaesthetizing the animals in magnesium chloride solution. A ring of body wall containing the sphincter muscle was cut out of the column below the tentacles and a few mm. above the attachment zone of the parieto-basilars. These rings were kept in running sea water until the muscle relaxed to a diameter almost equal to that of the original animal at rest. Small pieces of nylon gauze were then sewn round the ring on opposite sides to provide tags for anchoring and recording from the ring. Electrodes were placed against the fixed-position tag. When the preparation was fresh, single stimuli evoked quick contractions of the sphincters, which were very variable in magnitude (Fig. 9). Later, these responses became very much reduced in height and also slower, showing a summation on repetition at a slow rate. Following the delivery of eight to ten shocks at a rate of one per 5 sec. an additional response was elicited, appearing after a delay of a few seconds in some cases or roughly coinciding with the eighth or ninth shock (Fig. 10). This slow and smooth response resembles that obtained by Ross (1957*a*) from the isolated sphincter of *Calliactis*. The quick responses showed appreciable summation and facilitation effects (Fig. 11).

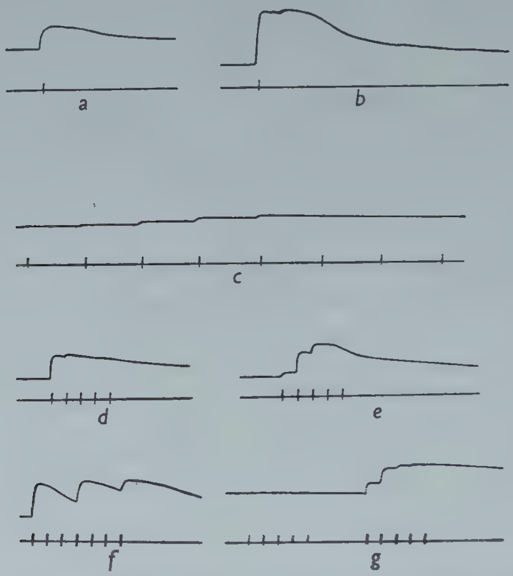

Fig. 9. Variable responsiveness of isolated sphincter muscle region of body wall. *a*, *b*, To single strong shocks; *c*, to weak shocks at one per 15 sec.; *d*, *e*, to single shocks at one per 5 sec.; *f* to paired shocks at two per 5 sec.; *g*, ageing preparation does not respond to single shocks but only to close pairs.

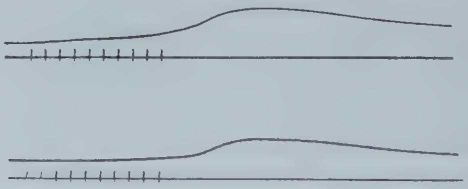

Fig. 10. Delayed slow response of isolated sphincter to weak shocks at one per 5 sec.

Elicitation of swimming responses. Preliminary tests using eight shocks delivered at one per sec. in order to test the Sund-Wilson finding were disappointing, giving swimming only occasionally and not consistently in the six animals tested. These animals showed no tendency to be more likely to respond after they had 'swum' once, or to respond subsequently to a smaller number of shocks. It was realized that to test the hypothesis adequately a large number of different animals of various sizes would have to be used. Wilson, in a private communication, tells me that the eighteen animals which were available to him were all small, with a disk diameter of about 2 cm. This corresponds to the smallest specimens available for the present experiments; most were 2·5–3 cm. or more in diameter, being preferable for recording. It is probably worth while, nevertheless, to record some of the observations made. Although the majority of anemones probably remained stationary throughout the summer, two specimens at least changed their positions. Of course, they could have moved by 'walking', but one animal, which was in a small glass dish awaiting its turn for experiment, was actually observed to swim, probably following a spontaneous take-off. It had been handled, but not for an hour previously; the temperature of the water had not exceeded 16° C. and there was no immediate source of irritation. Stephenson, it will be recalled, had noted 'jumping' by an animal in a small dish (as the temperature rose) and Sund (1958) noted the preliminary movements of 'swimming' when an animal became heated up under the lights used for filming; this animal, nevertheless, walked, not swam, away. But these scanty observations show that swimming might well occur spontaneously, or in response to a variety of stimuli. The response may well be a general one to adverse conditions, rather than a specific one to a particular species of starfish.

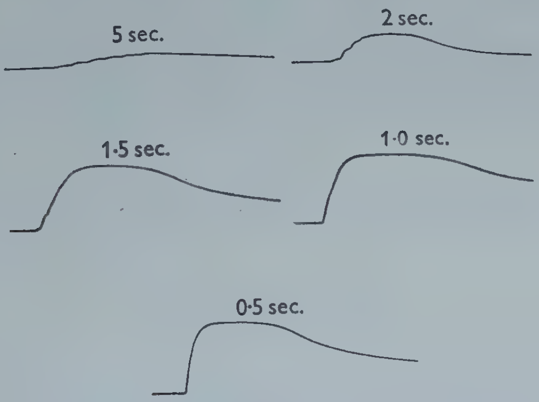

Fig. 11. Facilitation and summation in responses of isolated sphincter. Seven shocks applied at the intervals indicated.

Animals were stimulated electrically by applying the brief direct-coupled pulses through paired electrodes placed roughly mid-way up the column. The longitudinal retractor muscle response, which was probably that obtained by Sund and by Wilson as an initial response which preceded swimming, could be obtained in this position. The outstanding result of the experiments was simply the surprise engendered by their variability. One animal started to swim after only a single shock had been applied and went on to exhibit swimming movements without further stimulation (Fig. 11). The same animal could not be induced to swim by single electric shocks on later occasions. Other tests resulted in swimming in response to 5, 6, 7, 8, 9 or 10 shocks, or up to as many as 28 shocks, or no swimming at all. The 28-shock response (Fig. 12) occurred during a long series of shocks applied so as to evoke the parieto-basilar response and were delivered at the low rate of one per 10 sec.

More often than not there was an initial reaction, retraction followed by extension and waving, but without full swimming. As many of the animals used in these studies were tethered by pins they could not detach; a series of bendings following the initial reaction was taken to indicate that a full swimming reaction had occurred (Figs. 12, 13). The fact that these reactions occur at all, and continue for as long as normal free swimming, provides an important clue to the nature of the response. It cannot consist of a series of chain reflexes, for achievement of freedom from the attachment would surely constitute an important link in the chain of events.

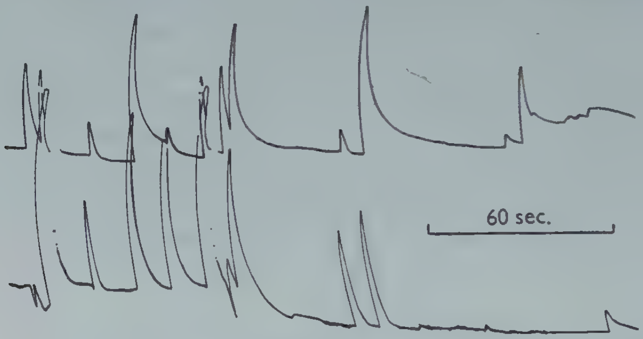

Fig. 12. Swimming movements elicited by a single very strong electric shock. Note absence of preliminary activities. Traces from opposite sides of animal.

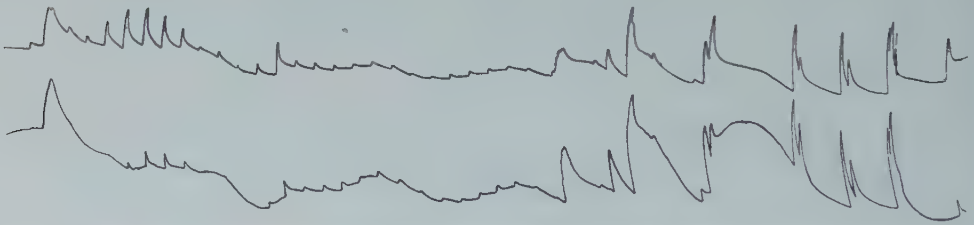

Fig. 13. Swimming movements elicited after twenty-eight shocks had been applied at one per 10 sec. Note initial part of swimming reaction after first shock. Swimming starts by a series of summating quick contractions; it is characteristic that these start not on the stimulated side (upper trace) but on the contralateral side. The great variability in the responses to the single shocks, due to the parieto-basilars, should also be noted.

Animals came to be recognized as 'swimmers' or 'non-swimmers', although a 'non-swimmer' might surprise one on occasions. Wilson (1959) states that stimuli applied at intervals greater than one per 3 sec. do not cause swimming, but during the course of some of the present experiments (responses of the parieto-basilars), full swimming movements occurred during repeated stimulation with single shocks at intervals as long as 10 sec., as in Fig. 13, though more than twenty stimuli were required.

Investigations on 'long-term facilitation'. The great variability encountered in the responses to stimulation aimed at evoking swimming made it impossible to use that approach extensively to test Wilson's finding of 'long-term facilitation'. The other methods suggested earlier were, however, extensively applied. They are:

first, testing the electrical threshold at intervals after first eliciting a motor response; secondly, testing the amplitude of the contraction to quantitatively similar stimulation applied at intervals after an initial response. These tests were applied to both the longitudinal retractor-muscle response and the parieto-basilar response. Wilson was probably arousing the longitudinal retractor response. But it is the parieto-basilar muscles, not the longitudinal retractors, which are used in swimming; this makes it difficult to see how the nerve-muscle junctions could be implicated. It would seem more probable that long-term facilitation would occur in the nerve net rather than at the nerve-muscle junctions as he suggested. Wilson was probably assuming that his electric shocks were exciting single impulse in a through-conducting nerve net, by analogy with Pantin's interpretation (1952) of the responses of the sphincter of *Calliactis*. Even if this is the case the excitation must in addition be affecting a great deal else, including sense organs and other parts of the nervous system, and these are likely to be affected in a much subtler manner.

Threshold changes

Examination of threshold changes would have no value as an experiment if the only reaction we expected was the simple one of eliciting a single all-or-nothing nerve impulse, but if there are also additional effects, on sense organs or nerve net, these might be affected differently from the observable motor response, and for them the shocks might well be suprathreshold. No effects were observed, however, as a result of giving shocks of a strength which just failed to evoke an observable motor response. There was no clearly detectable change in the subsequent response to suprathreshold stimuli, no increase in responsiveness to starfish; but the problem has not been tackled statistically, as it should be, owing to lack of time.

Amplitude of response

If the facilitation has occurred at sites between the excitable nerve elements and the muscles it should be detectable as an increased height of the response to a standard shock—single for the parieto-basilars, double for the longitudinal retractors. Once again it can be stated that if such effects occur at all they must be so small as to require a statistical test to detect them. Sometimes a facilitation was evident when the animals were tested soon after the initial stimulus, but this always died away after a few minutes. This effect could have been due to a relatively long-lasting 'arousal' of the nervous system, rather than to a persistent state of facilitation engendered at a particular set of junctions.

Reduced total number of shocks. Animals were also tested in the Wilson manner, by eliciting swimming and then applying a similar train of excitation but with one shock fewer. In none of these tests did a swimming response occur to the lower number of shocks, although there was usually some preliminary waving and occasional bending. Some animals could not be re-excited to swim at all for some time, and then only with a larger, not a smaller, number of stimuli than the original.

DISCUSSION

The failure, in all these experiments, to confirm the remarkably regular results obtained by Wilson is disappointing. Probably the difference must be attributed to differences in the animals. Wilson's specimens were all small and probably represented a very homogeneous group of young animals. If it should be confirmed that his results can be repeated with small, young animals, that will throw some interesting light on the changes which occur in the actinian nerve net as a result of maturation and experience, for the animals apparently become (untrainable) 'laws unto themselves' as they grow older, according to the indications of the present experiments. It is, nevertheless, hard to avoid the suspicion that the uniform way, for example, in which ten or so of Wilson's eighteen animals responded to precisely eight shocks was fortuitous. I find it difficult to imagine a mechanism by which a precise number of shocks could have such a well-defined meaning for a coelenterate nerve net, even an immature one, and be independent of frequency as a source of information to the net.

However, the fact that a non-biological stimulus in the form of a group of electric shocks can elicit the complex swimming reaction has been fully confirmed. The present interpretation, though, is that the swimming activity is a 'fixed-action' pattern, probably innate, which can occur spontaneously in response to certain extrinsic stimuli. I do not consider it to be a specific reaction to those starfish which elicit it and predict that other stimuli will be found which call it forth. It is indeed remarkable that so complex a reaction can occur in sea anemones at all. Where, and in what way, is the information required to activate the muscles sequentially stored in the nerve net? How is it called forth to elicit the phases of swimming, which take several minutes to complete? Since the sequence can be fully performed by tethered animals a chain-reflex interpretation cannot be applied to all stages. When the animal is cut in half whilst swimming (Sund, 1958; Robson, 1961) the halves of the animal continue to execute appropriate parieto-basilar contractions, so the integrity of the nerve networks of the whole animal is not essential for at least the later parts of the reaction. Robson (1961) has also shown that an isolated ring of body wall containing the tentacles and oral sphincter can react to a starfish by giving a prolonged contraction.

In considering the mechanism of initiation of swimming one is reminded of the way in which a variety of irritant substances increase the frequency of occurrence of responses to single shocks in *Metridium* (Ross, 1952). Rather randomly occurring impulses are evidently set up in the through-conducting nerve net by irritant chemicals, possibly by transmission across from another nerve net. It is visualized that in much the same way the secretions of certain starfish or electric shocks lead to impulses in a certain part of the nerve net which is caused, if the impulses persist long enough at a sufficiently high frequency, to evoke the sequence of nervous outflow which causes swimming. As the physiological state of the synapses between the nerve elements responsible is liable to be labile, it is not surprising that at different times different numbers of electric shocks or presentations of starfish should be needed to evoke the response, or that there are times, for any animal, in which swimming cannot be elicited. Furthermore, like any innate behaviour pattern the 'strength' of the response may vary, appearing as weak swimming, brief swimming or a show of only the preliminary phases of the reaction.

The failure, in the present experiments, to demonstrate a 'long-term facilitation' could be due to the present animals being older, having unadaptable synapses and so forth. Only a full-scale investigation on animals of different ages, accompanied by a statistical analysis of the data, is likely to provide acceptable evidence on this point.

Regarding the responses of specific muscles the experiments have made it clear that a through-conducting nerve network, similar to that demonstrated by Pantin in association with the oral sphincter of *Calliactis* and the longitudinal retractors of *Metridium* and also present in *Stomphia* in connexion with the longitudinal retractor muscles, is not the only excitable element which reacts to the electric shocks. In particular, the sensory elements and other parts of the nervous network are likely to be reacting. The great variability observed in the responses of the parieto-basilar muscles suggests that they occur indirectly via the motor nerve elements, and that impulses elicited by electric shocks must summate with those due to normal 'background' activity. The responses of most of the muscles are not similar to the superficially simple ones of the longitudinal retractors. It is not possible to interpret them on the basis that single impulses are set up in the appropriate motor nerve elements by single electric shocks.

SUMMARY

1. The spontaneous activity and also the response mechanisms to externally applied electric shocks have been studied in the swimming sea anemone *Stomphia coccinea* obtained from Puget Sound, Washington.

2. Slow rhythmical activity with a repetition interval of the order of 30 min. is evident in kymograph records of whole-animal movements and also in rings of body wall isolated from the oral or basal regions.

3. By suitably placing the stimulating electrodes different parts of the musculature can be brought into contraction. Those studied were principally the parieto-basilar muscles and also the longitudinal retractors, the circular muscles of the column and the isolated oral sphincter.

4. The magnitude of the response of the parieto-basilar muscles to constant-strength stimuli was very variable. Responses occur to single shocks and these increase in magnitude with increasing strength of stimulus.

5. The parietal muscles give delayed, slow contractions in response to trains of about ten stimuli at one per sec.

6. The isolated sphincter muscle shows two kinds of contraction, a quick one which occurs to single shocks and a slow one which occurs following a brief train of fairly widely separated shocks.

7. The ordinary circular muscles respond with a slow contraction to a few widely spaced shocks. They do not give quick contractions.

8. Although in many cases animals responded to eight quick shocks by giving swimming reactions this number was not found to be a precise requirement, and it varied for the same animal at different times in an unpredictable manner as well as varying from one animal to another. On one occasion a swimming response occurred after a single shock and on another after twenty-eight shocks.

9. It was found that the state of 'facilitation' (occurring in the response to electric shocks) of the muscles used in swimming varied a great deal but did not show any marked long-term change following a single shock or the elicitation of a swimming response.

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LOSS OF WATER BY EXCRETION AND DEFAECATION IN THE TSETSE FLY

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INTRODUCTION

Substantial amounts of water are excreted by tsetse flies immediately after the ingestion of a blood meal (Lester & Lloyd, 1928), and during subsequent stages of digestion faecal matter is voided in the form of a semi-liquid paste, a process which must also entail some loss of water. The water balance of tsetse flies cannot be satisfactorily elucidated unless such losses are taken into account, and the present investigation was undertaken in an attempt to provide quantitative estimates of the amounts involved.

It will be convenient to consider the preliminary excretion of water separately from the subsequent voiding of faecal matter, since the techniques appropriate to the study of these two processes are different. The present account will accordingly be divided into two sections.

I. THE EXCRETION OF WATER

Material and methods

The flies used for these experiments were *Glossina morsitans* Westwood, emerged from puparia which had been collected at Singida in the Central Province of Tanganyika. Within 2 hr. of emergence the flies were isolated in single 3 in. × 1 in. gauze-covered tubes and exposed to different relative humidities (80, 60, 40 and 20% R.H.) at 27° C. for 42 hr. They were weighed singly in glass-stoppered tubes on a Mettler balance reading to 0·01 mg., and then fed on the forearm of the experimenter in such a way that any excretion was retained in the glass tube and evaporation minimized. The tube was then re-weighed and the amount of blood ingested found by subtraction. Preliminary experiments had shown that the rate of excretion of water falls to a basal level in less than 3 hr. (see Fig. 1), and the flies were accordingly left for this period of time at 100% R.H. (to avoid loss by transpiration) and then re-weighed. The loss in weight was taken as the amount of water excreted. The size of the fly was then measured and the wet weight, dry weight and residual dry weight (dry weight minus fat) were determined separately for thorax and abdomen, all according to methods described elsewhere (Bursell, 1959, 1960). Knowing (*a*) the composition of blood (21·1% solids, see Hammarsten, 1914), (*b*) the amount of blood ingested, (*c*) the composition of the fly at the end of the experiment, and (*d*) the amount of water excreted during the experiment, the water content of the unfed fly could be calculated;* and in this way a relation could be obtained between the quantity of water excreted and the state of water reserves at the time of feeding.

The estimate arrived at will be subject to a number of systematic errors which must be briefly discussed:

(1) It is assumed that only water is lost by excretion during the first 3 hr. after a blood meal; in fact evaporation of the excreta to dryness leaves a deposit of crystals, but the amount was too small to be determined with the balance available, and constitutes on an average less than 0·1 % of the recorded weight losses.

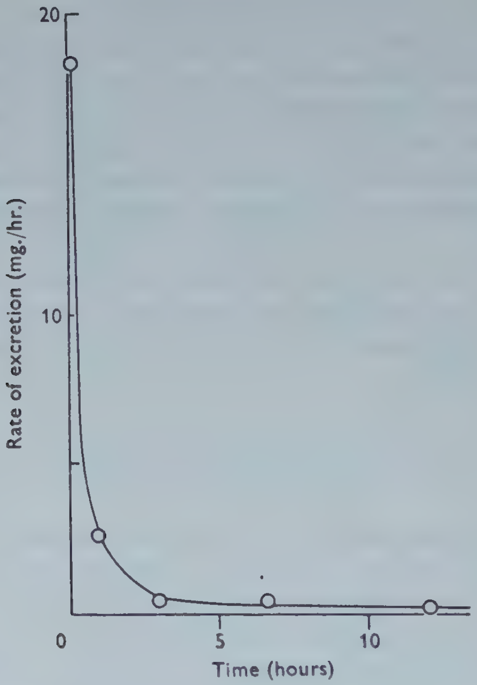

Fig. 1. The decrease in the rate of excretion of water after feeding. Each point represents the mean of six determinations.

(2) It is assumed that no loss of water occurs by transpiration, but in view of the difficulty of getting a truly saturated atmosphere this assumption also is questionable. However, even at humidities as low as 80 % R.H. the loss by transpiration would be less than 0·2 mg. for a 3 hr. period (Bursell, 1959) and negligible in comparison with the excretory losses which range from 10 to 20 mg.

(3) It is assumed that no loss in weight or change in composition is caused by respiratory metabolism. *G. morsitans* at rest at 27° C. consumes about 0·06 mg. of fat in 3 hr. with the production of an equivalent amount of water (Bursell, 1959).

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* The amount of water in the unfed fly would be the amount of water at the end of the experiment plus the amount excreted minus the amount ingested, and similarly for the amount of solid.

Again the quantities are negligible compared with the recorded losses, and in general it may be concluded that even in combination these systematic errors are too slight to affect the conclusions drawn.

RESULTS

Fig. 2 shows the amount of water excreted expressed as a percentage of the total blood meal, and plotted as a function of the water content of the feeding fly (amount of water as a percentage of the non-fatty wet weight). There is clearly a very strong correlation, the excretion being more copious the higher the water content of the fly. This is in accord with the early experiments of Jack (1939) who showed that what he termed the 'primary excretion' of flies exposed to low relative humidities was less than that of flies maintained at high relative humidities. It will be seen that in general about 40–50% of the water in the blood meal is excreted, a value which agrees with the figures quoted by Lester & Lloyd (1928) and by Jack (1939).

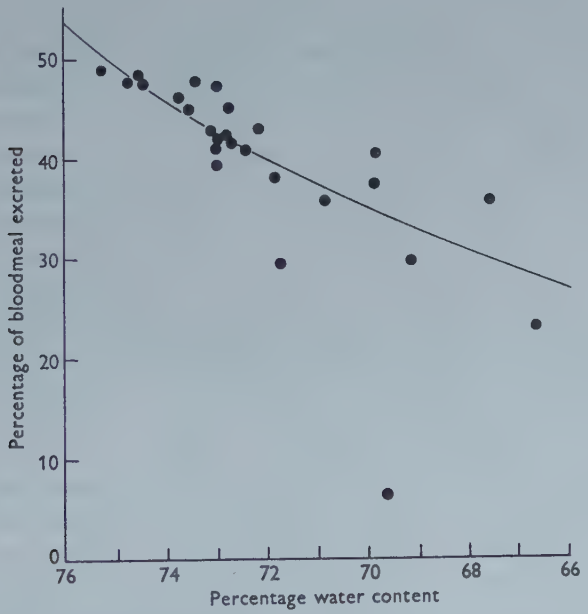

Fig. 2. The amount of water excreted (expressed as a percentage of the weight of the blood meal) plotted against the water content of the fly at the time of feeding. The curve has been calculated from the multiple regression, see text, p. 692.

For a quantitative assessment of the relation between water content and excretion it would clearly be desirable to express them both in the same unit. Water content was accordingly expressed in terms of 'tissue deficit', defined as the amount of water in mg. required to bring the tissues to the level of hydration characteristic of the newly fed flies, namely 75·6%, as determined from analyses of the isolated thorax. And to allow for the effect of size of blood meal, a multiple regression was calculated with blood meal (X_1 in mg.) and tissue deficit (X_2 in mg.) as the independent variables, and amount of excretion, in mg., as the dependent variable (Y). The corresponding formula was found to be

$$\hat{Y} = 0\cdot686X_1 - 1\cdot138X_2 - 4.151,$$

both regression coefficients being significantly different from zero ($s_{by_{2\cdot1}} = 0\cdot073$; $t = 9\cdot4$ and $s_{by_{1\cdot2}} = 0\cdot177$; $t = 6\cdot4$),* and 86% of the variance being accounted for by the regressions. The curve calculated from this formula, taking $X_1 = 28\cdot07$ mg. and the residual dry weight of flies as 5·158 mg., both mean values for the series in question, has been included in Fig. 2.

The most interesting result of this calculation is the close approximation of the regression coefficient $by_{1\cdot2}$ to unity (t for the difference from unity is 0·78, $P = 0\cdot4$–$0\cdot5$) which suggests that the excretion of water is accurately related to the state of tissue reserves—of the water which is removed from the blood meal an amount corresponding to the tissue deficit is retained in the body, and only the balance is excreted.

The value of the first regression coefficient ($by_{1\cdot2} = 0\cdot686$) is such that the dehydration of the blood meal appears to be dependent on the size of the meal. The water content of the blood meal at constant tissue deficit can be calculated from the data and it would be 56% for a full meal of 25 mg., but 65% for one of 15 mg. Since such an effect might be of significance in relation to water balance, in so far as it suggests that a greater proportion of water is retained in partial meals, it seemed worth while to investigate the phenomenon in greater detail and over a wider range of meal size.

The procedure employed was the same as before except that feeding of the flies was interrupted so that partial meals were taken. The dry weight of the tissues of the abdomen was estimated by subtracting the dry weight of the blood meal from the total as determined at the end of the experiment, and, assuming that the water content of the abdominal tissues is the same as that of the thoracic tissues, the amount of water held in abdominal tissues could be calculated. This, subtracted from the total amount of water contained in the abdomen would give the amount of water in the gut content, and, knowing the amount of solids in the gut, the water content of the blood meal could be calculated. Fig. 3 shows the water content 3 hr. after feeding plotted as a function of the size of the meal.† It is clear that the smaller the meal the smaller is the proportion of water which is removed from it. This effect seems to be independent of the tissue deficit of the feeding fly—a multiple regression was calculated with size of meal and tissue deficit as the independent variable, and the coefficient of regression with tissue deficit was found not to differ significantly from zero ($by_{2\cdot1} = 0\cdot502 \pm 0\cdot451$; t; 1·1; $P = 0\cdot3$).

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* The symbols, here and elsewhere, are those employed by Snedecor (1946).

† The amount of blood taken is expressed as a percentage of what would have been ingested by a fly of that size, had a full meal been taken. The estimates are based on a previously determined relation between the thoracic surface (see Bursell, 1960) of flies (X) and size of meal (Y), in mg. namely

$$\hat{Y} = 3\cdot65X - 3\cdot0.$$

The situation with regard to the excretion of water has been summarized in Fig. 4, of which curve (*a*) represents, for different sizes of meals, the amount of water in the blood ingested, and curve (*b*) the amount retained in the blood meal 3 hr. after feeding. The difference between these two curves represents the water available for replenishment of tissue reserves, the balance being excreted. 61% is the critical level to which water content may be reduced before death supervenes (Bursell, 1959) and for flies of the size of the present series this would correspond to a tissue deficit of about 10 mg. Fig. 4 shows that this amount of water would be available from a meal amounting to no more than 70% of a full one.

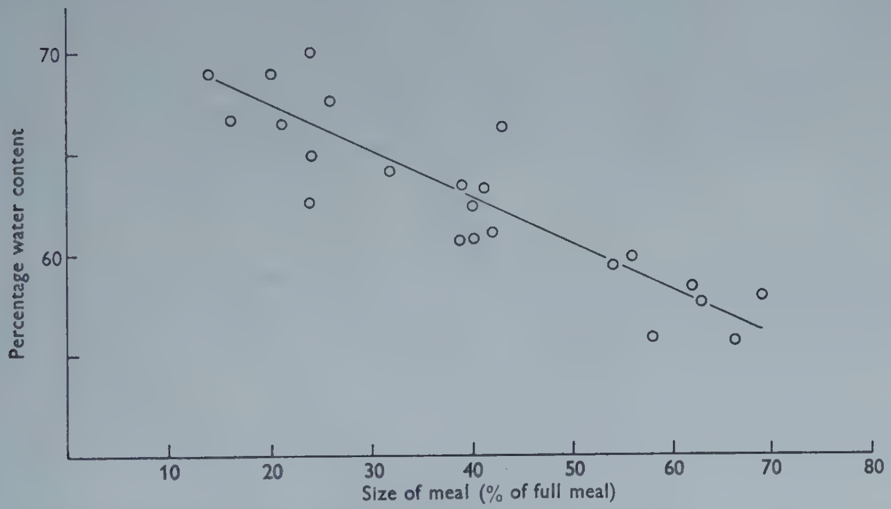

Fig. 3. The water content of the blood meal plotted against the size of meal taken (expressed as a percentage of the full meal). The line has been drawn according to the regression formula. $\hat{Y} = 71{\cdot}9 - 0{\cdot}227X$, where Y = water content, and X = size of blood meal. $Sy.x^2 = 0{\cdot}389$: $Sx^2 = 6361$.

The difference between curve (*b*) and the abscissa represents the amount of water retained in the blood meal, the dotted line showing what would have been retained had the percentage concentration been the same for all sizes of meal. When less than half of a full meal is taken the difference is as much as 1 mg. This retention of disproportionately large amounts of water after partial feeds may be of some importance to the fly, for 1 mg. represents about 10% of the total water reserves of the tissues.

II. THE EXCRETION OF WASTE PRODUCTS

Following concentration of the blood meal, nitrogenous waste materials begin to accumulate as a result of the deamination of products of digestion and general catabolic activity, and these, together with indigestible portions of the blood meal, mainly haematin, begin to be removed from the hind gut. The faeces are voided periodically as a semi-liquid paste, and it was of interest to determine how much water is lost with the faecal pellets, and whether the amount depends on the state of water reserves of the fly or on the humidity of the environment. The following procedure was adopted to investigate these questions.

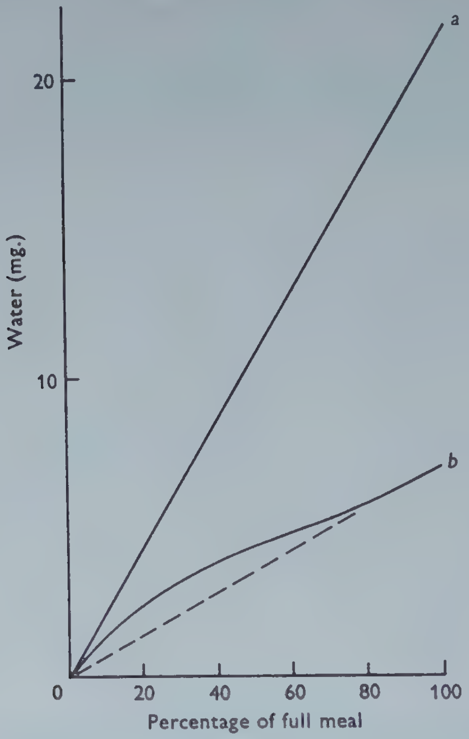

Fig. 4. The amount of water in freshly ingested blood (curve *a*) and in the concentrated blood meal (curve *b*) plotted as a function of the size of the meal. (For further explanation, see text.)

METHODS

Flies were fed and left for 3 hr. to complete the excretion of water, after which they were lightly anaesthetized with chloroform and the dorsal surface of the thorax was fastened to a copper wire holder by means of molten paraffin wax. A side piece of the holder passed to the ventral surface of the fly and to this were fastened the meso- and meta-thoracic legs to ensure that excreta were avoided freely and not picked up by the legs during the performance of cleaning movements. The fly was then mounted with plasticine in a glass container whose rim could be immersed below the surface of liquid paraffin contained in a petri dish. Humidity could be controlled in the closed space with wet filter-paper or silica gel, and loss of water from the faeces was prevented by collection under liquid paraffin.

Faeces were cleared at 08.00 and at 16.00 hr. each day; they were transferred by means of a wide-mouthed pipette to small watch-glasses made from heated cover-slips. Excess paraffin was quickly removed with a fine pipette leaving the faeces covered with a thin film. The watch-glasses were weighed and then exposed over phosphorus pentoxide at 37° C.; they were re-weighed at intervals to constant weight, the water content of the faeces being found by difference. The paraffin was now removed with benzene (30 min.) after which the faeces were dried *in vacuo* at 85° C. and the watch-glasses were reweighed. The faeces were removed with concentrated sulphuric acid, and the watch-glasses were washed in water, dried and weighed, the amount of solid material being obtained by subtraction.

Preliminary tests showed that known amounts of water added to watch-glasses under liquid paraffin could be quantitatively recovered in this way. Further it was shown that any change in weight of dry faecal material during a half-hour exposure to small volumes of benzene were too small to be detected with the balance available (Mettler reading to 0·01 mg.). As a further check on the method a mixture of uric acid, haematin and water was made up with a known proportion of solids, and its water content was estimated as outlined above. The values obtained were all within 3% of the true water content, a degree of accuracy which was considered sufficient for present purposes.

RESULTS

Fig. 5*a* shows the water content of faeces voided by *G. morsitans* maintained at 100% R.H. (curve (i)) and at 0% R.H. (curve (ii)). In saturated air the faeces first voided have a water content of about 60%, but values rise steadily in the course of the hunger cycle to reach levels of about 90% after 4–5 days. By contrast, the water content of faeces voided during desiccation in dry air is only about 40% during the first 16 hr., and drops a further 5% in the course of the hunger cycle (the flies maintained under these conditions die, presumably of desiccation, after about 3 days, while in saturated air flies will live for 5–6 days).

The fact that a substantial difference can be detected in the water content of faeces even at the first collection suggests that the difference might be a direct reflection of the difference in relative humidity, rather than in the state of water reserves; over the short period the water reserves of flies exposed to 0 and 100% R.H. would not differ very greatly (see Buxton & Lewis, 1934, p. 202; Jack, 1939, table XVIII; Bursell, 1959, table I). But further work would be required to settle this point.

Fig. 5*b* shows the output of solids; the rate appears to be slightly greater in dry air, but it declines more rapidly so that at the end of the third day the total amount of solid excreted is the same under the two sets of conditions, namely 2·5 mg. Taking average values of 72 and 35% for faecal water content in saturated and dry air the corresponding amounts of water lost during the first 3 days would be 6·4 and 1·4 mg. In other words, the ability to withdraw water from the faeces under conditions of desiccation represents a saving of 5 mg. of water, which is more than 30% of the total water reserves of the newly fed fly (10 mg. held in the tissues, see p. 693 above, and 6 mg. held in the blood meal, see Fig. 4).

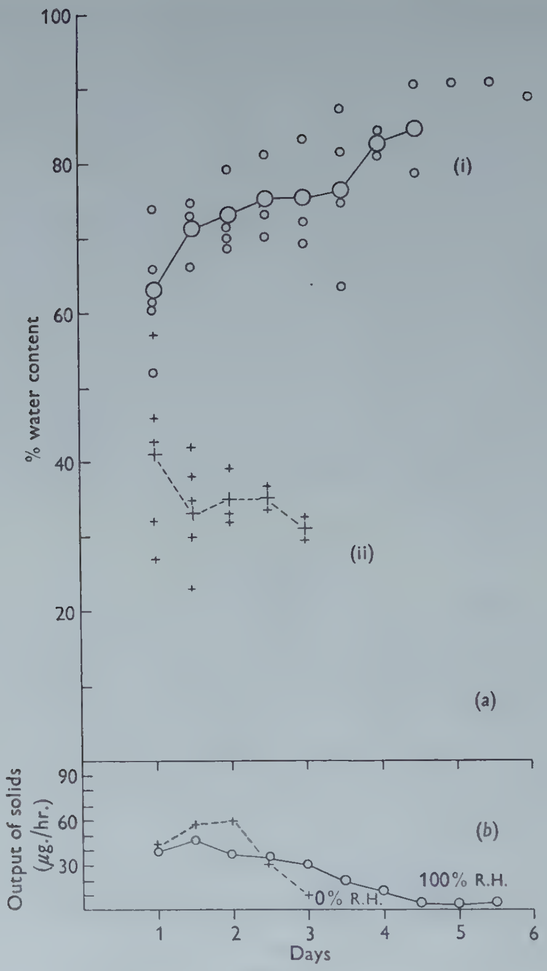

Fig. 5 (*a*) The water content of the faeces of flies maintained continuously at 100 % R.H. (curve (i)) and at 0 % R.H. (curve (ii)). Small symbols represent individual determinations, large symbols averages. The flies were fed at 12.00 hr. and the experiments started at 16.00 hr. on day 1. (*b*) The output of solid material of flies maintained at 100 % R.H. (○) and at 0 % R.H. (+).

DISCUSSION

It is well known that in many insects water is resorbed from the gut content in the region of the hind gut, and it seems that the rectal glands are particularly active in such resorption (Wigglesworth, 1950). Well-developed rectal glands, richly supplied with tracheae, are present in *Glossina* (illustrated by Hoare, 1931), and there can be no reason to doubt that these organs are largely responsible for the dehydration of faecal material. The interesting point which emerges from the present results is that although the mechanism is there, the tsetse fly does not at all times conserve water to the best of its ability; full powers of conservation are exercised only when water reserves are threatened. And this applies not only to excretory losses, but seems to be a general characteristic of the water balance of this insect. The control of transpiration by spiracular regulation in both adult and pupa finds full expression only if the humidity of the environment is low, or if the water reserves of the insect have been seriously depleted (Bursell, 1957, 1958). Since water conservation of either type would call for an expenditure of energy, it is possible that the phenomenon of regulated conservation should be seen as an instance of metabolic economy.

SUMMARY

1. The water content of the blood meal of tsetse flies is reduced from 79 to about 55% within the first 3 hr. after feeding.

2. The water which is abstracted from the gut contents serves to bring the tissues of the fly to full hydration, and any excess is excreted.

3. The degree of dehydration of the gut content depends on the amount of blood taken, relatively more water being retained in partial feeds.

4. The water content of faecal matter is about 75% in flies maintained at high relative humidity throughout the hunger cycle, only 35% in flies maintained in dry air. The difference reflects a saving of more than 30% of the total water reserves of the fly under conditions of desiccation.

Part of this study was carried out in East Africa under employment by the East African High Commission, and a preliminary account has appeared in the 1958 Annual Report of the East African Trypanosomiasis Research Organisation. I am indebted to the Wellcome Trust which provided a grant for equipment used to complete the investigations in Salisbury; and my thanks are due to Prof. E. B. Edney for helpful advice.

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THE SOURCE OF CARBON MONOXIDE IN THE FLOAT OF THE PORTUGUESE MAN-OF-WAR, *PHYSALIA PHYSALIS* L.

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INTRODUCTION

The Portuguese man-of-war is one of the most exotic creatures of the ocean surface. The gas-filled float which serves to buoy up some 30 ft. of tentacles stands well above the surface to catch the wind and attracts attention by its vivid blues, purples and greens. The float of the large Caribbean form is 12 to 18 in. long and contains a full litre of gas. The smaller animals which drift north in the Gulf Stream and on which the bulk of the present work was done contain some 300–500 ml. of gas in the float. Scientists have long been aware (Blainville, 1826, 1830, 1834; Lane, 1960) that the animal can replenish this gas. Haeckel (1888, p. 324), who had travelled extensively in southern seas to study the siphonophores, writes 'By compressing the float voluntarily, the animal can extrude the included air through the apical stigma and sink down. After a short time has elapsed it can rise again, secreting a great mass of gas by the pneumadena, and filling the float. I often observed this process repeated, in December 1866, off the Canary Islands.'

The gases found accumulated in the float are clearly derived in large part from air. Schloesing & Richard (1896) record for two pooled samples of gas 0·0 and 1·7 % CO_2, 12·2 and 15·1 % oxygen, the balance being nitrogen and argon. Argon and nitrogen were present in the same proportion as in air, indicating that these gases entered the float from the air. In addition to the components derived from air, carbon monoxide has been found to comprise a significant proportion of the total gas volume in the floats of animals carried north in the Gulf Stream and captured off Martha's Vineyard Island in Massachusetts (Wittenberg, 1958).

The present experiments establish that the isolated gas gland or pneumadena forms carbon monoxide *in vitro* when supplied with L-serine. Carbon monoxide, it is suggested, probably serves to inflate the float and is later slowly replaced by air through diffusion and exchange.

METHODS AND RESULTS

The gases accumulated in the float

Carbon dioxide, oxygen and non-absorbable gases were determined in the float gases by the gasometric method of Scholander, van Dam, Claff & Kanwisher

(1955). Carbon monoxide was determined gasometrically by absorption with a solution of cuprous chloride in ammonium chloride (Wittenberg, 1960); or, when present in low concentration, estimated colorimetrically (Shepherd, 1947).

Carbon monoxide present in the float gases was identified by the concordance of the volumetric and colorimetric analyses and by the characteristic carboxyhaemoglobin colour and spectrum exhibited by haemoglobin solutions equilibrated with the float gases.

Table 1. *Float gases of* Physalia

Float		Gas composition	
Length (in.)	Volume (ml.)	O_2 (%)	CO (%)
2	9	19·6	1·4
3	15	15·1	12·7
4	23	14·9	12·6
4	28	18·9	3·5
5	32	17·0	6·7
3	35	19·5	0·4*
5	39	16·5	12·3
4	44	19·4	1 *
5	49	16·8	10·3
6	52	14·9	11·8
5	63	16·4	12·6
4	69	18·9	4·5
6	96	17·4	9·1
6	108	19·3	3·7
7	116	18·7	2·9
6	127	18·4	6·1
6	132	18·9	3·3
7	157	17·5	0·5*
6	159	17·8	7·4
6	167	19·4	0·4*
7	168	17·8	6·2
7	170	18·9	5·0
7	185	18·2	2·6
7	186	19·0	2·0
7	187	18·2	4·9
7	190	19·2	3·2
5	251	21·2	2·0
7	254	19·0	4·5
5	281	19·6	1 *

* Colorimetric analysis (Shepherd, 1947).

The float gases of fifty individuals captured during the late summers of 1957, 1958 and 1959 at Woods Hole, Massachusetts, contained an average of 16% oxygen (range 13–20%), and an average of 2% carbon monoxide (range 0–11%). An approximate measurement of total combustible gas showed no combustible gas other than carbon monoxide. These specimens had of course come far from their native subtropical seas and were in relatively cool waters when captured. It was felt that a truer picture of the normal float gases would be obtained from animals captured in their native environment. In Table 1 are presented the compositions of the float gases of animals obtained near Miami, Florida, in February and March 1960. I am grateful to Mrs Peter Wangersky who obtained these specimens. It will be seen that the carbon monoxide content is higher than in the specimens from Massachusetts and is often near 10%. The oxygen content of the float gases of animals from both areas is less than in air, suggesting a metabolic consumption. The carbon dioxide content of all specimens was less than 1%.

The site of carbon monoxide production

Tissues dissected from recently captured *Physalia* were incubated for 20 hr. at 23° C. in 30 ml. portions of sea water contained in 50 ml. syringes. The syringes were agitated frequently during the early hours of incubation. At the end of the incubation period, about 25 ml. of air was drawn into the syringe and any carbon monoxide present was extracted into the gas phase by vigorous shaking. The gas phase was transferred to a second syringe, the extraction with air was repeated, and the air extracts were combined. Because of the limited solubility of carbon monoxide in water, this procedure should extract essentially all of the carbon monoxide present. Carbon monoxide in the air extracts was estimated colorimetrically by the method of Shepherd (1947). The air is passed through a glass tube containing silica gel as a desiccant and a portion of silica gel is treated with palladium sulphate and ammonium molybdate.* The palladium-catalysed reduction of the molybdate leads to the production of a green colour which is estimated by comparison with standards.

The zoöxanthellae, symbiotic algae living in the outer layer of the float, the pneumatocodon, were considered to be a possible source of carbon monoxide. On this assumption, numerous attempts were made to demonstrate carbon monoxide formation by the green and non-green portions of the pneumatocodon, both in the light and in the dark, in the presence and in the absence of serine. No carbon monoxide production was detected in the pneumatocodon. Tentacles were not tested. Carbon monoxide was formed, however, by the inner layer of the float, the pneumatosaccus. The data presented in Table 2 establish that in the presence of D,L-serine as substrate carbon monoxide production is sharply localized in the specialized area of the pneumatosaccus known as the gas gland or pneumadena. Large bubbles of gas could be seen to form around the gland fragments during the early hours of the incubation.

The gas gland of these large, 7–8 in. long, animals weighed about 0·2 g. The pneumatosaccus, of course, is very much larger than the gland and this fact should

Table 2. *The site of carbon monoxide production*

(The tissues were incubated in 30 ml. of 0·02 M D,L-serine in sea water for 20 hr. at 23° C.)

	Carbon monoxide formed (ml.)	
Animal no.	Gas gland	Pneumatosaccus
A	0·2	0·000
B	0·4	0·000
C	0·4	0·005
D	0·2	0·000
E, F	0·1	0·000

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* Indicating tubes for M-S-A Carbon Monoxide Tester, Catalogue No. DS 47134, Mine Safety Appliances Company, Pittsburgh 8, Pa.

be borne in mind in interpreting the data of Table 2, since the entire non-glandular part of the pneumatosaccus was incubated.

The substrate for carbon monoxide production

Dissected gas glands were divided in quarters and the fragments from several animals were distributed at random among a number of 50 ml. syringes so that each syringe received four fragments. 30 ml. portions of sea water containing dissolved substrates were added and the syringes were incubated 20 hr. at 23° C. Carbon monoxide formed was estimated as before. No carbon monoxide formation was detected with the following solutions of substrates prepared by adding to sea water concentrated solutions adjusted to the pH of sea water: 0·01 M D-lactic acid plus 0·01 M pyruvic acid; 0·02 M 2-ketoglutaric acid; 0·01 M L-aspartic acid plus 0·01 M L-glutamic acid; 0·02 M glycine; 0·02 M D,L-alanine; 0·02 M D,L-threonine; 0·02 M D,L-methionine; 0·05 M methanol; 0·02 M-NaCN. 0·02 M formic acid supported carbon monoxide formation to a limited extent.

Serine was found to be the substrate for carbon monoxide production. The data presented in Table 3 demonstrate the support of carbon monoxide production by L-serine. D,L-Serine was also effective. D-Serine was not tested.

Table 3. L-*Serine the substrate for carbon monoxide production*

(Gas glands were divided into halves and the halves incubated separately in either 30 ml. sea water or 30 ml. 0·01 M L-serine in sea water for 20 hr. at 23° C.)

	Carbon monoxide formed (ml. per gas gland)	
Animal no.	Sea water	0·01 M serine in sea water
G	0·02	0·2
H, I	0·02	0·2
J	0·15	0·4
K	0·00	0·1
L	0·04	0·2
M	0·1	0·4
N	0·1	0·4
O	0·04	0·4
P	0·02	0·2

In the intact animal the gas gland probably receives a continuous supply of nutriment from the main bulk of the stem on which it rests. In the experiments the gland is, of course, deprived of its source of food. In an attempt to replace this supply, gas gland fragments were incubated in 0·02 M D,L-serine solution containing either 0·01 M L-aspartic acid plus 0·01 M L-glutamic acid or 0·5% casein hydrolysate* or fragments of pneumatocodon. In no case was there any increment in carbon-monoxide production over that found with serine alone. Oxygen may quite possibly be required for the formation of carbon monoxide, and it should be noted that the total supply of dissolved oxygen in the syringe was only 0·16 ml. and that the access of the tissue to this supply was limited by inadequate stirring.

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* Tryptic hydrolysate of casein: N-Z-Case, Sheffield Chemical Co., Norwich, New York.

Some components of the gas gland

The gas gland appears faintly coloured to the eye and stands out in contrast to the surrounding pneumatosaccus which is water-white. The colour of the gland is not well defined but extracts are faintly pink. Water extracts of the gland exhibit intense absorption maxima at 260 and 305 mμ and a very much weaker maximum at 510 mμ to which the visible colour of the gland probably is referable. The absorbency, calculated for a layer of glandular tissue 1 cm. thick and relative to water, is 80 at 305 mμ and is 2·1 at 510 mμ.

The reported rapid secretion of gas by *Physalia* implies a very high concentration in the gas gland of the chemical system responsible. It might be anticipated that co-factors utilized by the enzyme system would also be present in high concentration. *A priori* vitamin B_6 (pyridoxine, pyridoxamine, pyridoxal and the corresponding phosphates) might be expected to be required for the activation of serine, and folic acid might similarly be required for the manipulation of the one-carbon fragments which are ultimately transformed to carbon monoxide. Analyses of the gas gland and of the pneumatosaccus for these vitamins and for two vitamins which are probably not involved in carbon monoxide formation are presented in Table 4. Vitamin analyses were carried out by Dr Maria Burger of the Wisconsin Alumni

Table 4. *Vitamin contents of the gas gland*

(μg. per g. wet weight.)

	Tissues			
	Gas gland		Pneumato-saccus‡	
Vitamin	1*	2†		Method
Folic acid	23·8	23·7	0·10	*A.O.A.C.* 1955, p. 830
Folinic acid	2·45	2·45	0·07	Sauberlich & Baumann, 1948
B_6	2·74	2·78	0·15	Atkins *et al.* 1943
Thiamine	—	3·4	0·47	*A.O.A.C.* 1955, p. 819
Riboflavin	—	0·50	0·18	*A.O.A.C.* 1955, p. 823

* Pool of four glands.
† Pool of six glands.
‡ Pool of twelve animals, gas gland not included.

Table 5. *Vitamin contents of some mammalian tissues*

(μg. per g. wet weight.)

	Vitamin			
Tissue	Folic acid	B_6*	Thiamine†	Riboflavin†
Beef liver	2·9 ‡	6·5	2·6	33
Beef kidney	0·58‡	6·7	3·7	26
Beef heart	0·03‡	2·4	5·8	8·9
Beef muscle	0·1 ‡	2·8	0·7	1·5
Rat liver	1·83§‖	—	—	—
Rat kidney	2·50§‖	—	—	—
Chicken liver	3·77‡	—	—	—

* Snell & Keevil (1954).
‡ Stokstad (1954).
‖ Snell (1950).
† Spector (1956).
§ Mitchell & Isbell (1942).

Research Foundation. For comparison, Table 5 lists the vitamin contents of several animal tissues selected for their high content of folic acid. It will be seen that the vitamin B_6, folinic acid, thiamine and riboflavin contents of the gas gland are in no way remarkable. The folic-acid concentration on the other hand is very high, and is roughly ten times as great as that found in the mammalian tissues. One may infer that folic acid forms a part of the carbon monoxide-producing system and that this system is present in the gland in very high concentration.

DISCUSSION

The present work has established that the carbon monoxide found in the float gases originates in the structure known as the pneumadena or gas gland. This structure has been described (Haeckel, 1888, p. 342; Chun, 1887, p. 559) as a thin layer of ectodermal glandular cylindrical cells. It is necessary to inquire whether the gas gland is indeed responsible for the elaboration of the float gases. The question cannot be answered directly because it has not as yet been studied in *Physalia*. However, Jacobs (1937) has observed the rapid replacement of gas in the floats of the siphonophore *Stephanomia bijuga*. The gas was not analysed. Jacobs noted that the gas unquestionably arose from a structure described as a gas gland and located in the 'funnel' at the base of the simple float of this animal. Chun (1887, p. 559) has traced the embryological development of the gas gland of *Physalia* from a simple structure very similar to that of *Stephanomia* to the highly modified, asymmetric, flattened and expanded structure found in the adult. It is reasonable to conclude that the structure in *Physalia* which is called the gas gland is homologous with the gas gland of siphonophores of simpler structure and is therefore, in all probability, actually responsible for the secretion of gas.

Of great interest in this regard is the extraordinary development of the gas gland described by Haeckel (1888) in siphonophores captured aboard H.M.S. *Challenger* from the depths of the ocean. In these forms the gas gland is massively developed and is housed in a new structure, the aurophore, which provides a greatly increased area of contact between the gland cells and the gas phase of the float. Although the depth of capture cannot be known with certainty, the recorded depths, 640 and 516, 275, 600, fathoms for *Stephalia*, *Stephonalia* and *Rhodalia*, respectively, correspond to hydrostatic pressures of 50–120 atm., and the animals must be capable of secreting gases into the float in the face of these pressures.

The nature of the gases secreted by *Physalia* into the float can only be known by direct analysis of the secretion product of the gas gland, since the accumulated gases may be assumed to have exchanged material with the atmosphere. Unfortunately, the author has not had an opportunity to observe the re-inflation of the float or to analyse the newly secreted gas.

Carbon monoxide clearly is a component, athough possibly not the sole component, of the secreted gas. Although the rates of carbon monoxide formation observed *in vitro* were small, they were certainly minimal since the tissue was deprived of a source of nutriment and had a very limited oxygen supply. On the other hand, the very large concentration of folic acid encountered in the gas gland would imply that the gland is capable of converting L-serine to carbon monoxide very rapidly. Secretion of carbon monoxide, followed by exchange of gases with the atmosphere and metabolic consumption of oxygen, can account for the observed composition of the accumulated gas.

Carbon monoxide accumulation occurs but seldom in nature. The only other example known to the author is the accumulation which has been noted in the hollow stems of marine algae (reviewed in Blinks, 1951) where it may comprise 5 % of the total gas. The source of this carbon monoxide is not known.

SUMMARY

1. Carbon monoxide, 0·5–13 %, is found in the float gases of *Physalia*. Oxygen comprises 15–20 % of the total gas. Carbon dioxide is present in negligible amounts.

2. The gas gland, pneumadena, is the site of carbon monoxide formation.

3. L-Serine is the substrate for carbon monoxide formation.

4. A strikingly large concentration of folic acid is found in the gas gland.

5. It is suggested that carbon monoxide secretion serves to inflate the float of *Physalia* and that carbon monoxide is later slowly replaced by air through diffusion and exchange.

This investigation was supported by a Senior Research Fellowship (S.F. 57) from the United States Public Health Service and by a Public Health Service research grant (H 3719) from the National Heart Institute, Public Health Service.

My special thanks go to the members of the Supply Department of the Marine Biological Laboratory at Woods Hole who went to great efforts to capture most of the *Physalia* used in this study; and to Mrs Peter Wangersky for obtaining the samples of gas from animals at Miami, Florida. The skilled assistance of Mrs Martin Schiffer is gratefully acknowledged.

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SOME FACTORS AFFECTING THE OXYGEN CONSUMPTION OF *ASELLUS*

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(*Received* 3 *May* 1960)

INTRODUCTION

Asellus aquaticus is often abundant in polluted streams (Allan, Herbert & Alabaster, 1958) and under certain conditions its oxygen consumption may be of significance in studies of oxygen balance. In an earlier paper (Edwards, 1958), the need for comparative studies, using different respirometer systems, was stressed. The experiments described in the present paper were carried out using both a Warburg constant-volume respirometer* and an apparatus in which the fall in dissolved-oxygen concentration of water passing through a respirometer was measured, using a polarographic technique.

Most of these experiments have been confined to a study of the effect of body size, temperature, and oxygen concentration on the oxygen consumption of *A. aquaticus*. Some observations have also been made on the diurnal rhythm of *A. aquaticus* and the effect of oxygen concentration on the oxygen consumption of *A. meridianus*, a species which has not been recorded in organically polluted streams.

An extension of this study is not possible at the present time and the authors apologize for the incomplete nature of certain aspects of the work.

MATERIALS AND METHODS

All experiments were conducted between December and March except where the time of year is specifically mentioned.

A. aquaticus was collected from three locations:

(i) Maple Lodge Sewage Effluent Channel, Rickmansworth, Hertfordshire (G.R. SU 039910). The average dissolved-oxygen concentration at the collection site for the year 1953–54 was about 40% of the saturation value and during the winter months was below 30% (Allan *et al.* 1958).

(ii) River Hiz, Henlow, Bedfordshire (G.R. TL 190378). Animals were collected from an area 4–5 miles below Hitchin Sewage Works. Oxygen records are not available for this site but data have been collected for an area near the effluent discharge (Gameson & Griffith, 1959) and here the average dissolved-oxygen concentration was about 50% during the winter of 1957–58.

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* These experiments were carried out by Mr R. W. Edwards, whilst on the Staff of the Freshwater Biological Association.

(iii) River Ivel, Stotfold, Hertfordshire (G.R. TL 223373). Collections were made in this unpolluted and spring-fed chalk-stream about 3 miles from its source. In November 1958 a polarographic dissolved-oxygen recording machine was placed at the collection site and the average dissolved-oxygen concentration was between 70 and 80 % for the winter period and did not fall below 60 % (Department of Scientific and Industrial Research, 1960, Fig. 41).

A. meridianus was collected from a pond in Baggrave Park, Leicestershire (G.R. SK 696086).

Animals were kept in the laboratory at 20° C. (± 2° C.) in aerated water for at least 24 hr. after collection and were allowed to feed, except in certain Warburg experiments described later where animals were starved for 24 hr. before oxygen-consumption measurements were made. Animals used in experiments at 10° C. were acclimatized to that temperature for 24 hr. Respirometers were fitted with hoods to exclude light. The wet and dry weights of groups of animals were determined, but there was no change in the proportion of dry matter in these groups (about 20 %) with an increase in size. The effect of moulting on the wet weight: dry weight ratio in individual specimens was not studied.

Warburg respirometer

The techniques used were similar to those described in an earlier paper (Edwards, 1958). *A. aquaticus* from the Sewage Effluent Channel was used exclusively and experiments were conducted between December 1956 and March 1957 except for those on diurnal rhythms, which were conducted in July 1957.

Polarographic respirometer

Polarographic techniques have been employed to measure oxygen concentrations in respirometer studies for many years (Berg, 1953). Some workers have described techniques which measure the dissolved-oxygen concentration continuously (Mann, 1958; Bielawski, 1959). The apparatus used in the present study measures the concentration of dissolved oxygen in the water before and after it has passed through the respirometer tube and records these concentrations on a strip chart.

General description. A general diagram of the apparatus is shown in Fig. 1. Water passes from the 20 l. reservoir *A*, through a constant-level device *B*, to a 10 l. reservoir *C*, where it is maintained at a constant temperature and the content of oxygen is controlled by saturation with a predetermined mixture of air and nitrogen. From the reservoir *C* water passes either (*a*) by direct route, *D*, to a two-way tap *G* which controls the flow through the apparatus; or (*b*) by route *E*, through the respirometer U-tube *F*, to tap *G*. Both routes may be closed near the reservoir *C* by screw clips. From the tap *G*, water passes through the electrode chamber *J*, to a flowmeter *K*, and then to waste. When water is passing through the direct route from the reservoir, water may also pass through the respirometer route by opening tap *H*. The water flow in this route is controlled by tap *H* and measured at the flowmeter, *I*. By including an alternative route in the respirometer line the dissolved-oxygen concentration in the direct line (and hence reservoir *C*) can be recorded and at the same time a similar flow of water can be maintained through the respirometer route so that animals may be kept under controlled oxygen and flow conditions throughout the experiment.

The proportions of air and nitrogen in the gas mixture are controlled by the needle valves *Q*. Pressure-reducing valves *P* maintain a gas supply at constant pressure to the needle valves, and flow meters *R* measure the gas flows. The two gas streams are mixed in the chamber *S* before passing through a diffuser *T* in the reservoir. Provision is made for drawing off water samples from the reservoir for the analysis of oxygen concentration by the Winkler method by a siphon tube *V*.

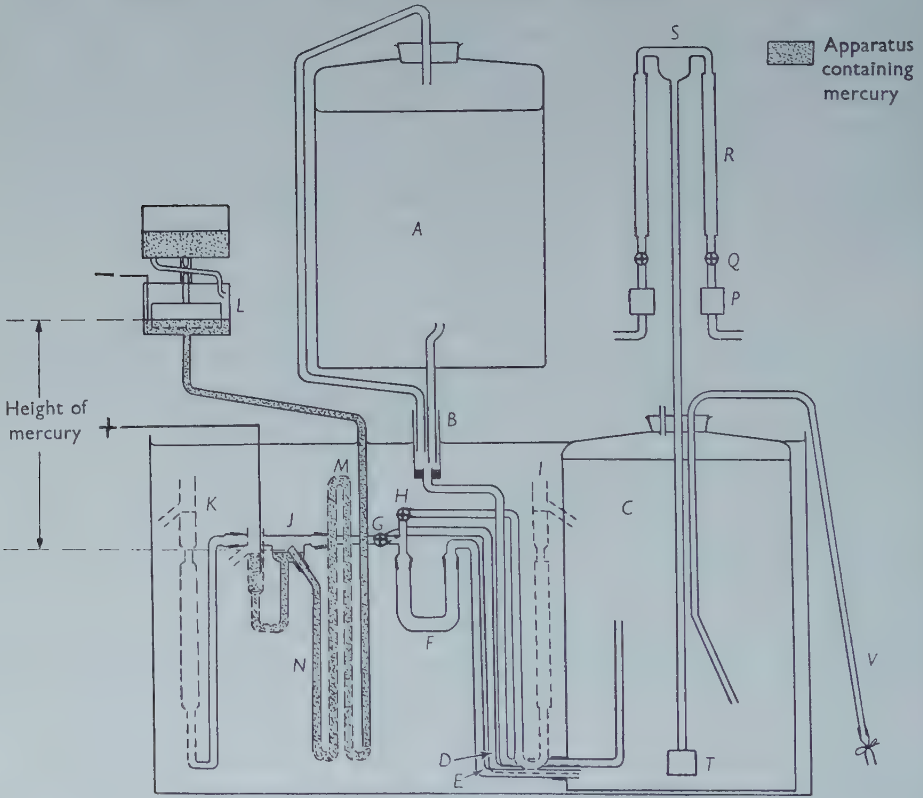

Fig. 1. General diagram of polarographic respirometer. Items drawn in dotted lines are attached to the outside of the constant-temperature bath.

Polarographic analysis of dissolved-oxygen concentrations. A wide-bore dropping-mercury electrode was used, similar to that described by Briggs, Dyke & Knowles (1958) with a mercury pool reference electrode. The mercury drop rate was controlled by the height of the mercury reservoir *L* above the electrode tip (24 cm.) and two lengths of capillary tubing, 60 cm. of 0·2 mm. bore (*M*) and about 40 cm. of 0·8 mm. bore (*N*).* The dropping-mercury electrode was kept at 1·65 V.

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* For details of mercury level control and pre-treatment of capillary tubing see British Patent Application No. 40151/59.

negative with respect to the mercury pool. The system operates on the second oxygen plateau (Kolthoff & Lingane, 1952). In initial experiments slow current drifts were caused by the deposition of calcium carbonate on the tip of the dropping-mercury electrode. Addition of 3 p.p.m. (as P) of sodium hexa-meta-phosphate to the water prevented this and high stability, lasting several days, was achieved. The water used in the experiments was unchlorinated tap water with a total hardness of about 290 p.p.m. (as $CaCO_3$). Fig. 2*a* shows a typical calibration curve for the apparatus; unlike those of Berg (1953), Mann (1958), and others, this is not quite linear. Fig. 2*b* shows polarograms of the second oxygen plateau from which this calibration curve was constructed. The dissolved-oxygen concentrations were calculated from the saturation values of Truesdale, Downing & Lowden (1955) and the partial pressure of gases in the gas mixture, and were checked by Winkler analysis of bottle samples from the reservoir *C*. Unless different potential differences had been applied at different dissolved-oxygen concentrations, a truly linear relation between current and oxygen concentration could not have been achieved. The electrode system was not temperature-compensated and all experiments were conducted at 20° C. (±0·1° C.). Unlike the systems of Bielawski (1959) and Mann (1958), the current readings were independent of flow between 60 and 170 ml./hr. The position of the dropping-mercury electrode and the design of the electrode chamber are important in determining the dependency of current readings on flow rates. All experiments were conducted at flows of 100–130 ml./hr.

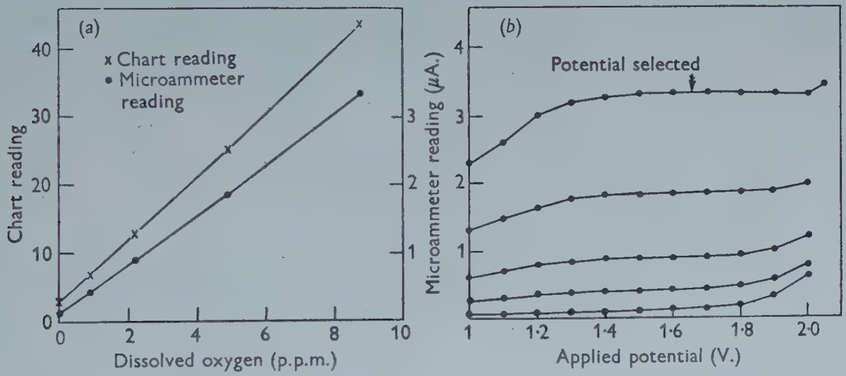

Fig. 2. Calibration graph and polarograms of polarographic respirometer.

The electrical circuit is shown in Fig. 3. Provision was made either to record the current produced by the polarographic cell on a strip chart, readings being taken every 3 min., or to read it directly on a microammeter. The microammeter in conjunction with a voltage control was useful in providing a quick check of the characteristics of the second oxygen plateau (see Fig. 2*b*). A standard cell giving a current of 5·4 micro-amp. was built into the circuit to check recorder sensitivity, but is not shown in the circuit diagram.

Procedure. Animals were placed in the respirometer and water was passed through both direct and respirometer routes at a predetermined rate, the dissolved-oxygen concentration of water in the direct route passing through the electrode chamber being recorded. Tap *H* was then closed and tap *G* revolved, allowing water from the respirometer route to pass through the electrode chamber. The dissolved-oxygen concentration in water leaving the respirometer was recorded for at least 1 hr. and the flow measured. Finally, the stability of the dissolved-oxygen concentration in the reservoir was checked by returning to the direct route and recording the reading. This routine was repeated at different dissolved-oxygen levels (see Fig. 4). After the gas mixture had been changed it took about 1 hr. before stable readings were achieved.

RESULTS

Warburg experiments

Fig. 5 shows the logarithmic relation between oxygen consumption per unit weight and wet weight of *A. aquaticus* at 10 and 20° C. Larger animals were separated into males and females* and certain experiments were conducted with animals which had been starved for 24 hr. Results of analysis of covariance suggest that there is no significant difference in the oxygen consumption between the two sexes or between fed and starved animals (i.e. between centres of gravity of groups shown

Fig. 3. Electrical-circuit diagram of polarographic respirometer.

*A*1	Ammeter, F.S.D. 3 A.
*A*2	Microammeter, F.S.D. 5 μA., resistance approximately 3000 Ω.
B	12 V. accumulator.
*C*1	5000 μF. electrolytic condenser. 6 V. working.
D.M.E.	Wide-bore dropping-mercury electrode.
Hg pool	Reference electrode (mercury pool in polarographic cell).
M	Microswitch operated by a cam which is driven by a synchronous clock motor. Contacts close for several seconds every 3 min. (Synclock motors from Everett Edgcumbe & Co. Ltd., Colindale Works, London, N.W. 9).
P.P.	12 V. power pack—could be replaced by a standard battery charger capable of delivering about 3 A. at 12 V.
*R*1	9 Ω, 18 W.
*R*2	50 Ω, 3 W. (wire-wound).
*R*3	190 Ω, ¾ W.
*R*4	2500 Ω, 3 W. (wire-wound).
RC	Recorder solenoids for printing and moving chart (chart solenoid operates on release). They are part of *REC*.
REC	Cambridge Model B 'thread recorder' modified by manufacturer to give solenoid-operated print and chart-movement. F.S.D. 5 μA., resistance approximately 2000 Ω. (Cambridge Instrument Co. Ltd.)
RECT	Full-wave rectifier, Sentercel type B84-1-1 W. (Standard Telephone and Cables Ltd., Edinburgh Way, Harlow, Essex.).
*S*1	Double-pole on-off toggle switch.
*S*2	Double-pole change-over toggle switch.
*S*3	Double-pole change-over toggle switch.
T	Heavy-duty filament transformer with all secondaries connected in series and capable of delivering 3 A. at 18 V. (Radiospares, 4, Maple Street, London, W. 1).
V	Voltmeter, F.S.D. 3 V.

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* Females with brood pouches were not used in these experiments.

in Fig. 5). Although the relation between size and oxygen consumption is similar for fed and starved animals ($P = 0{\cdot}6$ at 10° C. and $P = 0{\cdot}9$ at 20° C.), it may be different for the two sexes ($P = 0{\cdot}05$ at 10 and 20° C.). Regression analyses on grouped data gave coefficients of $-0{\cdot}32\ (\pm 0{\cdot}03)$ at 10° C. and $0{\cdot}28\ (\pm 0{\cdot}02)$ at 20° C. The oxygen consumption per unit weight is proportional to these powers of the wet weight.

A. aquaticus consumes oxygen about 1·5 times as fast at 20° C. as at 10° C. over the size range studied and results suggest that the Q_{10} value is not dependent upon size, the difference in regression coefficients being insignificant ($P = 0{\cdot}3$).

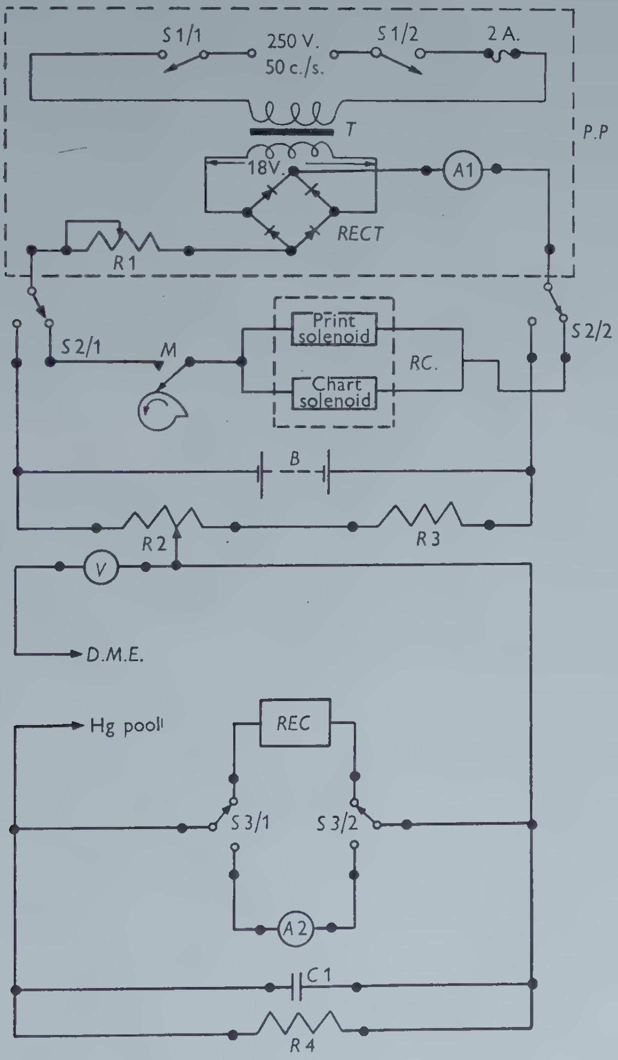

Fig 3. For legend see opposite page.

The oxygen consumptions of several groups of *A. aquaticus* were determined at four times the normal amplitude of shaking of the respirometer flasks. The difference between groups was not significant ($P > 0{\cdot}5$).

Lang & Ruzickova-Langova (1951) reported a diurnal rhythm in the oxygen consumption of *A. aquaticus*, throughout the year, the lowest oxygen consumption being recorded around noon. It is not clear whether the animals were exposed to constant or variable illumination, however, and experimental observations were continued for only 9 hr., generally between 9 a.m. and 6 p.m. Fig. 6 summarizes observations made in the present study. At six-hourly intervals groups of males were placed in the dark in respirometer flasks and their oxygen consumptions were measured for 24 hr. The interpretation of these data is difficult, but it seems that there is an initial decline in oxygen consumption during a period of acclimatization, and that there is a daily rhythm of small amplitude even under constant environmental conditions, with a minimum oxygen consumption around noon. A more general decline has been reported by Fox & Simmonds (1933). They found that the oxygen consumption on the second day of measurement in a Barcroft respirometer was 93% of that on the first day. The high initial rate observed in Groups II, III and IV (see Fig. 6) was not observed in Group I. This is probably due to the coincidence in Group I of the acclimatization period (with its high oxygen consumption) with a period of minimal daily oxygen consumption. The experiments discussed earlier in the paper correspond to Group I animals in that observations started at 9 a.m. and no systematic fluctuation was observed.

Polarographic experiments

Initial experiments showed that no systematic changes occurred in the oxygen consumption of *A. aquaticus* for at least 12 hr. in this apparatus under constant conditions. The coefficients of variability between hourly averages over this period were about 7%. In view of the similarity of the oxygen consumption of males and females described above, sexes were not separated for these experiments; the larger size groups were exclusively male, however. Such a grouping may be criticized on two counts: first, the oxygen consumption of males and females may be differently affected by size, and secondly, the level of oxygen consumption may be different when sexes are grouped. Fig. 7 shows the logarithmic relation between oxygen consumption per unit weight and wet weight of *A. aquaticus* in air-saturated water at 20° C., data from three areas having been grouped. The oxygen consumption per unit weight is proportional to $-0{\cdot}22\ (\pm 0{\cdot}07)$ power of the wet weight. The effect of size on the oxygen consumption is not significantly different from that calculated earlier for the Warburg respirometer ($P \simeq 0{\cdot}3$); the level of oxygen consumption determined in the polarographic respirometer is, however, significantly higher ($P < 0{\cdot}001$).

It will be seen from Fig. 8 that the reduction in oxygen consumption between dissolved-oxygen concentrations of about 8·3 and 1·5 p.p.m.* is small in all groups.

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* The average of the respirometer influent and effluent concentrations has been taken throughout this paper. Generally the conditions, for example, flow and animal numbers, were arranged so that a fall of about 1 p.p.m. could be expected.

The oxygen consumption is expressed as a percentage of that determined at the beginning of the day in air-saturated water. Values above 100% were sometimes obtained when animals were returned to air-saturated water at the end of a day's observation.

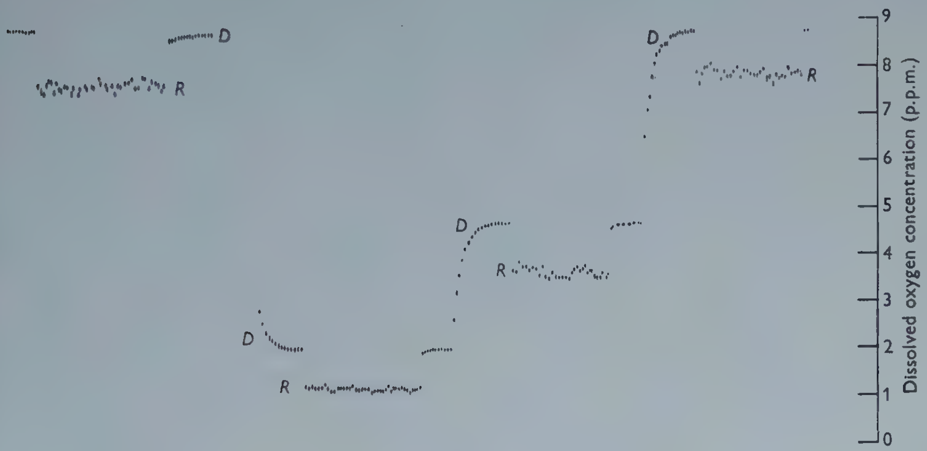

Fig. 4. Typical recorder chart showing oxygen consumption of *A. meridianus* at 20° C. at three oxygen concentrations (recordings were made every 3 min.). Flows at these oxygen levels were not identical and therefore the oxygen consumption is not directly proportional to the fall in oxygen concentration as it passes through the respirometer. Direct route = *D*. Respirometer route = *R*.

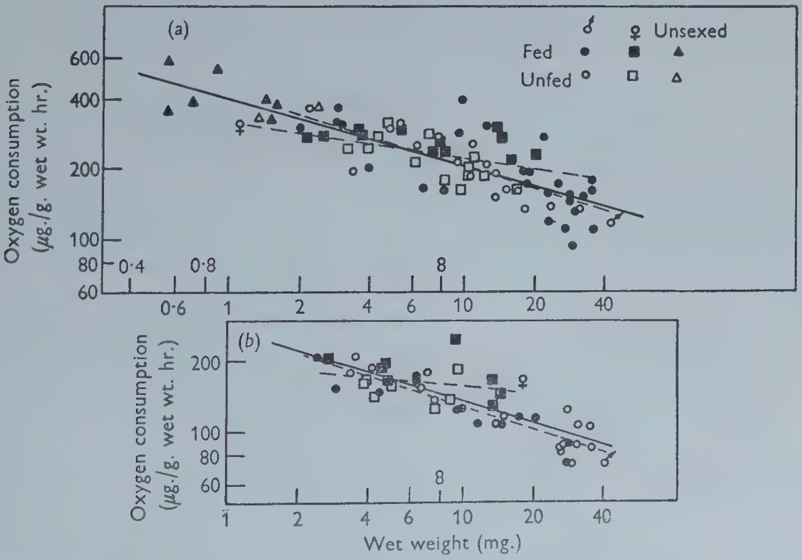

Fig. 5. Log oxygen consumption per unit weight plotted against log wet weight for *A. aquaticus*. The continuous lines are drawn for grouped data and dotted lines for the separate sexes. (*a*) at 20° C., $b = -0{\cdot}281$; (*b*) at 10° C., $b = -0{\cdot}321$.

A close inspection of charts (Fig. 4) reveals differences in the variability of the dissolved-oxygen concentration of the effluent from the respirometer at different oxygen levels. At high dissolved-oxygen concentrations the concentration in the effluent is variable and would be even more so were it not for mixing in the tubing and electrode chamber. At low oxygen concentrations the effluent concentration is

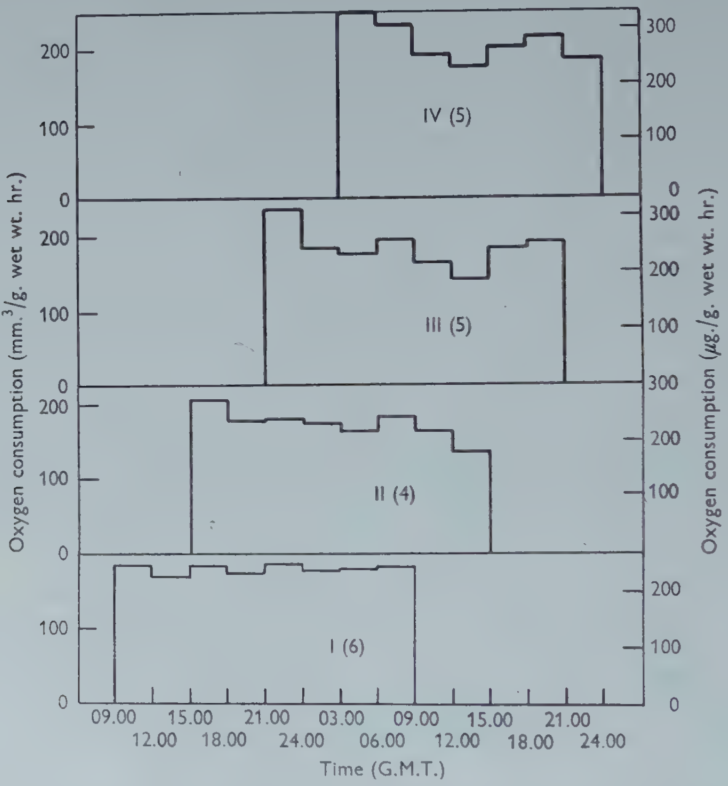

Fig. 6. Oxygen consumption of *A. aquaticus* at 20° C. over 24 hr. Figures in parentheses indicate the number of groups for which the average oxygen consumption has been taken.

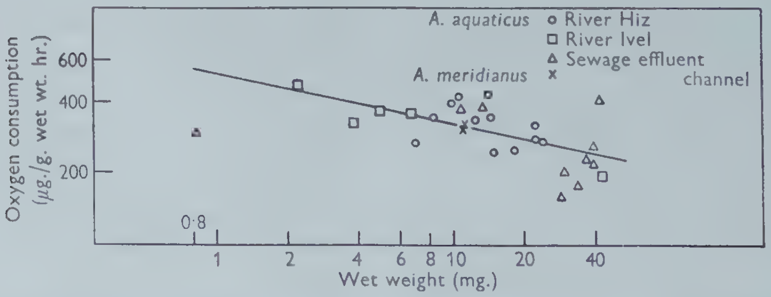

Fig. 7. Log oxygen consumption per unit weight plotted against log wet weight for *A. aquaticus* and *A. meridianus* measured in the polarographic respirometer. Temperature 20° C., $b = -0{\cdot}22$.

relatively constant. It seems likely that the greater variability in oxygen consumption is due to periods of locomotory activity. In the confined space of the respirometer, movement by one animal tends to disturb others and promote a more general activity, resulting in a period of high oxygen consumption. Variations in the oxygen consumption of trout observed by Job (1955) were shown to be caused by irregular swimming activity. At low oxygen concentrations locomotion is reduced and pleopod movement becomes more regular. The effect of dissolved oxygen concentration on pleopod movement has been studied by Fox & Johnson (1934).

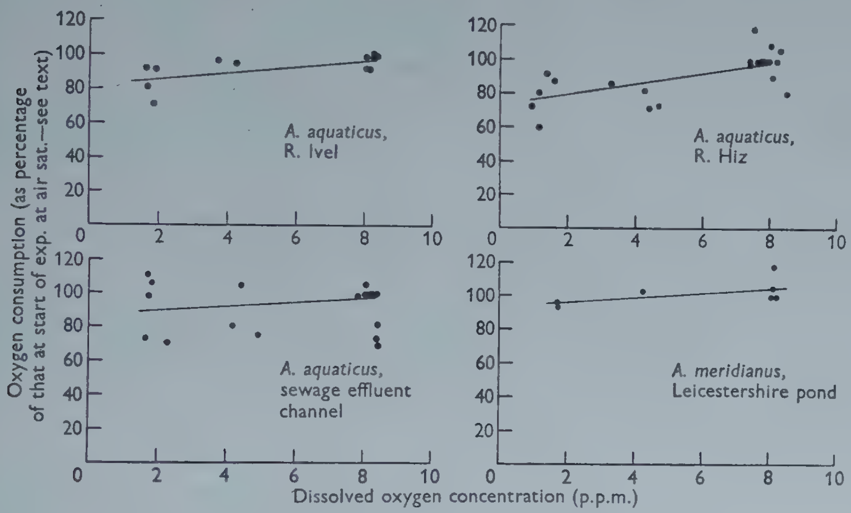

Fig. 8. Oxygen consumption of *A. aquaticus* and *A. meridianus* from different habitats at different dissolved-oxygen concentrations.

DISCUSSION

Fig. 9 shows data on the oxygen consumption of *Asellus* species collected from various sources. Fox & Simmonds (1933) reported differences in the oxygen consumption of anaesthetized *A. aquaticus* from a fast- and a slow-flowing stream. All the streams from which animals were collected for the present study were slow-flowing, with velocities generally less than 1 ft./sec. Other habitat differences, for example, dissolved-oxygen levels and degree of pollution, did not influence oxygen consumption.

Sprague (in preparation) showed that *A. intermedius* respires at similar rates at 10 and 20° C. in a Warburg respirometer after acclimatization to these temperatures for one week. In the present study, *A. aquaticus* respires 1·5 times as fast at 20 as at 10° C. after temperature acclimatization for only 1 day.

Will (1952) found the oxygen consumption of *A. aquaticus* at 23° C. in a darkened Warburg respirometer to be about twice that measured in the present investigation at 20° C. in the Warburg respirometer. The temperature difference of 3° C. between the two series would seem inadequate to account for a difference of such magnitude and no other explanation can be offered.

Allee (1929) measured the oxygen consumption of *A. communis* males in bottles, analysing oxygen concentration by the Winkler method. His data have been grouped and plotted in Fig. 9. *A. communis* clearly reaches a much greater size than the other species discussed, but assuming that its oxygen consumption/size relationship is comparable, its oxygen consumption is very similar.

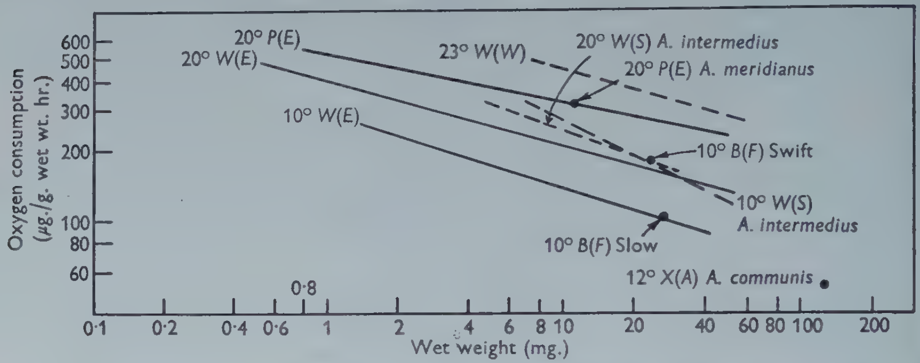

Fig. 9. Log oxygen consumption per unit weight plotted against log wet weight for species of *Asellus*, collected from various sources (Allee, 1929, (*A*); Fox and Simmonds, 1933, (*F*); Sprague, in preparation, (*S*); Will, 1952, (*W*) and present authors (*E*)). The respirometers referred to in the figure are Warburg, *W*; Barcroft, *B*; Polarographic, *P*; and Bottle, *X* (see text). Data refer to *A. aquaticus* wherever species are not given.

The differences between the oxygen consumption as measured in the polarographic and Warburg respirometers are probably associated with intensities of activity under rather different experimental conditions. Although sexes were not separated in the polarographic respirometer experiments it seems unlikely that the grouping could account for this difference, which extends over the whole size range; in both series the larger size-groups were exclusively male. Animals moved freely in both respirometers and neither gives a measure of the 'basal metabolic rate'. A comparative analysis of activity in these respirometers has not been attempted in the present study.

The oxygen consumptions of *A. meridianus* and *A. aquaticus* are very similar at 20° C. and both species behave similarly in low oxygen concentrations. It would be rash to assume, from these experiments, that the distribution of these species is not influenced by the oxygen conditions of their habitats. The effect of oxygen concentration on activity, growth, reproduction, etc., would need to be studied before any ecological generalization could be made concerning the influence of oxygen concentration on distribution.

The oxygen consumption of *A. aquaticus* and *A. meridianus* may be described as of the 'regulatory' or 'independent' type between oxygen concentrations of 8·3 and 1·5 p.p.m., the oxygen consumption between these two concentrations being reduced by only 15 to 20%. It seems probable from recorder-chart characteristics and other evidence that locomotory activity decreases and pleopod activity increases (Fox & Johnson, 1934) as the oxygen concentration falls. Differences in activity pattern have been described more fully for *Chironomous plumosus* (Walshe, 1950), where the proportion of time spent in feeding decreases and that spent in respiratory irrigation increases below 3 p.p.m. Similar changes in ventilation activity have also been described for *Phryganea grandis* (Van Dam, 1938). The assumption that an 'independent' type of oxygen consumption is favourable to an organism (Berg & Ockelmann, 1959) or implies maintenance of full metabolic function is invalid wherever such changes in activity pattern take place that the 'scope for activity' (Fry, 1947) decreases. Animals with such a behaviour pattern can survive only relatively short periods of exposure to low oxygen concentrations unless they can also withstand longer periods of starvation. In the case of *A. aquaticus*, Warburg measurements suggest that the oxygen consumption was not reduced after starvation for 24 hr. Sprague (in preparation) has shown that *A. intermedius* can withstand oxygen concentrations as low as 0·3 p.p.m. for about 7 days at 20° C. when no food is available.

SUMMARY

1. The oxygen-consumption rates of *Asellus aquaticus* (males and females) have been measured at 10 and 20° C. using a constant-volume respirometer, and the effect of starvation for 24 hr. investigated. The oxygen consumption is approximately proportional to the 0·7 power of the wet weight. The rate of oxygen consumption at 20° C. is greater than at 10° C. by a factor of 1·5.

2. The oxygen-consumption rates of *A. aquaticus* and *A. meridianus* have been measured at 20° C. in a flowing-water respirometer employing a polarographic technique for the measurement of dissolved-oxygen concentrations. The oxygen consumptions of *A. aquaticus* and *A. meridianus* are similar and decrease by 15–20 % when the dissolved-oxygen concentration falls from 8·3 to 1·5 p.p.m.

3. The oxygen consumption of *A. aquaticus* is between 35 and 75 % higher in the polarographic respirometer than in the constant-volume respirometer.

We wish to thank Prof. H. P. Moon (Leicester University) for his interest and help in supplying *A. meridianus* and Dr J. B. Sprague (Fisheries Research Board of Canada) for his unpublished data on the oxygen consumption of *A. intermedius*. Mr G. Knowles, Mr R. Briggs and Mr W. H. Mason provided much help and advice in the construction of the polarographic respirometer.

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THE REFLEX CO-ORDINATION OF THE HEART BEAT WITH RESPIRATION IN THE DOGFISH

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INTRODUCTION

The linking of the heart beat with respiration was first noted in *Torpedo* by Schoenlein in 1895. He described a 1:1 co-ordination, a pattern reported by later workers in other elasmobranchs (Lyon, 1925; Lutz, 1930*a*). Lutz (1930*b*) demonstrated that in dogfish the cardiac vagus was concerned in this co-ordination but otherwise the phenomenon has received little attention. In this paper branchiocardiac co-ordination and the mechanism effecting it will be described from the results of experiments on forty specimens of *Squalus lebruni* Vaillant. The major findings from this species have been confirmed on six specimens of *Mustelus antarcticus* Gunther. Finally, the suggestion will be advanced that this co-ordination of two systems jointly concerned in supplying oxygen to the tissues enhances the efficiency of this process.

MATERIAL AND METHODS

Mounting

Fish intended to provide information on branchiocardiac co-ordination were not anaesthetized, and were restrained by two clamps to the dorsal fins, in a rectangular tank filled with running sea water from the supply tanks of the University Marine Station. The fish weighed from 3½ to 6 lb. and no system of artificial perfusion was used. Sea-water temperatures varied from 4° C. in winter to 16° C. in summer.

When surgical procedures were intended, such as the cannulation of blood vessels, the fish were anaesthetized by 3 min. immersion in 1:1000 tricaine (MS. 222, Sandoz). They were then rigidly mounted underwater in a metal frame of the type described by Satchell & Restieaux (1958). In the few experiments in which curare was used the fish was artificially perfused through the spiracle.

Recording

Most of the records were made on an Ediswan 4-channel pen writer. The electrocardiogram (E.C.G.) was recorded with pre- and post-cordial leads consisting of insulated needles inserted through the body wall to lie ventral to the heart; signals from these were fed into a conventional E.C.G. amplifier. Respiration was recorded from two fine brass contacts clipped into the upper and lower jaws. When the jaws closed a 10 mV. pulse was fed into a second E.C.G. amplifier.

In some experiments brief inflations of the pharynx were made with a small air-filled balloon connected by a spiracular cannula and polythene tube to a hand bulb and also by a side arm to a tambour pressing against a strain gauge, this last being connected through a d.c. amplifier to the pen writer.

Blood pressure was recorded from the first branchial artery (lateral ventral aortic pressure) and from the coeliac artery (lateral dorsal aortic pressure). Both arteries were cannulated with 1 in. long No. 17 hypodermic needles connected by 61 mm. lengths of 2 mm. P.V.C. tubing to two Statham P 23 AA pressure transducers. The transducers connected in this way have a response better than 0–22 cyc./sec. Their outputs after d.c. amplification were fed to the two beams of a Dumont 5 SP 11 A oscilloscope and photographed with a Grass model C 4 D kymograph camera.

Branchial blood flow was monitored by a photo-electric method. Light from a 6 V. bulb was passed through the gill on to a Philips OCP 71 red-sensitive photo-transistor tube, the output of which was displayed on the oscilloscope after d.c. amplification. Electrical stimuli were provided by a Grass square-wave stimulator.

The elasmobranch Ringer's fluid of Keynes & Martins-Ferreira (1953) was used to moisten nerves and to act as a diluent of such drugs as were used; injections were made into the anterior cardinal sinus.

RESULTS

The electrocardiogram

The E.C.G. of elasmobranch fish has been described by Kisch (1948); it is here used only to signal the heart beat and will not be described in detail. The shapes of the various deflexions vary considerably with minor changes in electrode placement; Fig. 1 A shows a typical trace. With pre- and post-cordial leads the *P* wave was either upright or inverted; it signals the onset of auricular contraction. The *QRS* deflexion was large; it signals the invasion of the ventricle by the cardiac impulse and the *P–R* interval thus corresponds to the time taken by the impulse to cross the auricle and the auricular-ventricular junction. At 12–16° C. the *P–R* interval was 0·3–0·4 sec. The *T* wave occurs as the ventricle starts to repolarize; it signals the end of the contraction; the *P–T* interval at 12–16° C. was 1·2–1·4 sec. The small excursion between *QRS* and *T* has been shown by Kisch (1948) to be due to the contraction of the conus.

The respiratory record

The pen writer records each respiration as an upstroke when the mouth opens and a downstroke when it closes. A respiratory cycle is considered as starting when the mouth opens and for the purpose of analysis has been divided into ten phases of equal duration (Fig. 1 B upper scale). From phases 1 to 7 the mouth is open, the gill slits are closed and the pharynx is dilating as it fills with water. Just after the start of the 8th phase the mouth closes and remains closed to the end of the cycle. During phases 8–10 the water in the pharynx is expelled from the gill slits. The time of most rapid water flow across the gills has been determined with the same pressure-recording device (Statham P 23 AA) as was used for recording blood pressure. The water-filled cannula was placed so that its opening was within the 4th gill pouch. In this situation the outflowing water current was registered as a change in pressure at the cannula tip. In Fig. 1 B this trace was recorded on the oscillograph together with the mouth movement. It can be seen that the pressure recorded in this position reached a peak at the division between the 9th and 10th phases of the respiratory cycle.

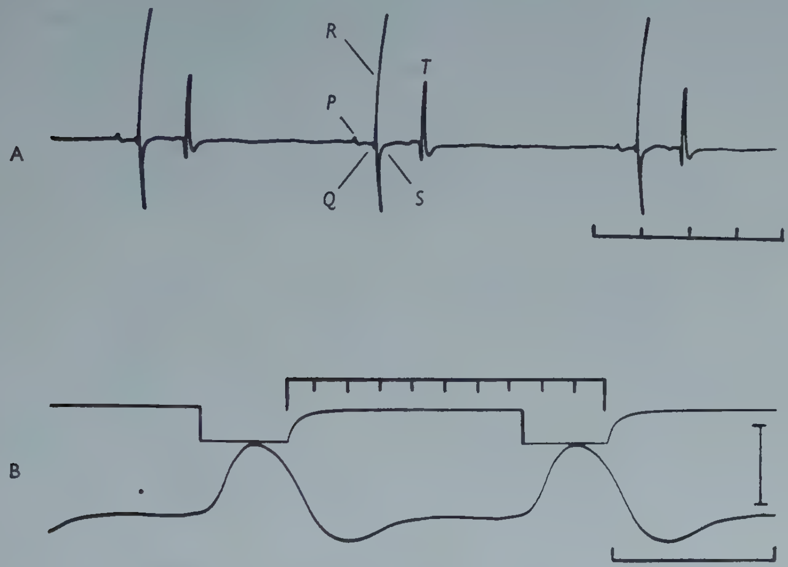

Fig. 1. A, E.C.G. of *S. lebruni*, 11° C. The deflexions have been given the conventional designations. B, Oscilloscope display of respiration. Top beam: mouth closure: opening = upstroke. Vertical strokes retouched. Lower beam: water pressure at gill opening indicating rate of expulsion of water across gills. Time marks in A and B = sec. Upper scale in B shows tenths of a respiratory cycle. Calibration = 1 cm H_2O.

The phenomenon of co-ordination

In all fish studied in this series the heart rate was lower than the respiratory rate; two patterns of co-ordination normally occurred. In some fish the interval between heart beats was consistently some simple multiple of the length of the respiratory cycle; rhythms of 1:2 (Fig. 2A), 1:3 or 1:4 were observed for more than a hundred successive beats. *Mustelus antarcticus* provided some particularly striking runs of unchanging rhythm. More commonly the heart made a succession of beats at one multiple followed by a delayed beat at the next higher multiple of respiratory cycle length. The following sequence taken from *Squalus lebruni* is typical. (A space has been arbitrarily introduced after each longer beat.)

3334 3334 33334 3334 3334 33334 3334 33334 334 3333334 33334
33334 34 334 3334 334 34 3334 34 334

The advent of the longer cardiac cycle cannot be predicted; its occurrence, no doubt, depends on the constantly fluctuating background of vagal tone, which is itself presumably related to the changing pattern of visceral and somatic stimuli impinging on the central nervous system. Lutz (1930*b*) has shown that a wide variety of stimuli, both visceral and somatic, inhibit the heart beat. Fig. 2B is an example of alternating 1:2, 1:3 rhythm.

There is some indication (Figs. 2A and B) that the heart was beating at a particular phase of the respiratory cycle, the *P* wave tending to occur either just before (phase 10), or just after (phase 1), mouth opening. The point is better demonstrated in Table 1. Here one hundred consecutive heart beats from each of eleven fish (1 *Mustelus antarcticus*, 10 *Squalus lebruni*) have been placed in the phase of respiration in which they occurred. The totals for the ten *S. lebruni* analyses have been added together and presented as a histogram (Fig. 3), which is thus a summary of the occurrence of 1000 heart beats from ten fish recorded at temperatures from 4–16° C. The co-ordination is manifested as a statistical trend—a tendency for the heart to beat immediately after the opening of the mouth. Phase 7, just prior to mouth closure, is the period in which the heart is least likely to beat, only 1·8 % of the *P* waves originating at this time. Of thirty fish analysed in this way eighteen possessed this pattern of co-ordination. It has been accepted as 'normal' and others regarded as atypical for the following reasons.

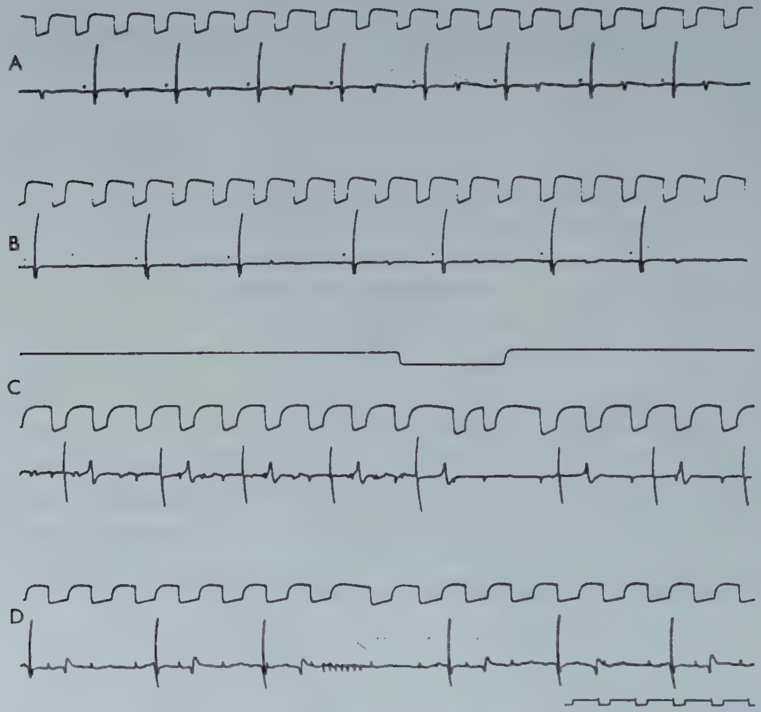

Fig. 2. Co-ordination in *S. lebruni*. All at 16° C. A and B. Upper trace: respiration; upstroke = mouth opening. Lower trace: E.C.G. The *P* wave is insignificant in this trace and its position is indicated by a dot. C. Upper trace: inflation of pharynx with a balloon; inflation downwards. Middle trace: respiration. Lower trace: E.C.G. The closing of the mouth switch makes a small down-going artifact in this experiment. D. Respiration and E.C.G. with upgoing respiratory switch artifact. After 3rd *T* wave, central end of cut branchial branch of the left vagus stimulated with 7 square wave 1 msec. 2 V. pulses. Shock artifact downwards. Time = sec.

Table 1. *Number of heart beats in a series of 100 occurring in each tenth of a respiratory cycle from eleven fish* (*ten* Squalus lebruni, *one* Mustelus antarcticus)

Species	T.	Phase of respiratory cycle									
		1	2	3	4	5	6	7	8	9	10
M.	10	26	7	1	—	—	—	—	—	4	62
S.	4	42	13	—	—	2	1	1	4	9	28
S.	8	29	22	16	8	1	1	2	1	4	16
S.	10	38	23	8	10	2	3	1	3	3	9
S.	10	24	22	20	11	10	6	—	1	—	6
S.	10	19	12	7	6	2	1	—	12	15	26
S.	10	33	29	15	6	5	2	—	—	1	9
S.	11	31	21	13	9	8	4	—	1	3	10
S.	15	22	8	9	11	6	11	7	6	4	16
S.	16	18	16	6	2	4	4	3	2	11	34
S.	16	20	9	7	2	1	—	4	7	20	30
Total		276	175	101	65	41	33	18	37	70	184

T. = temperature in ° C., M. = *M. antarcticus*, S. = *S. lebruni*

The atypical patterns vary widely, ranging from fish showing no co-ordination at all, in which approximately equal numbers of heart beats occur in each phase of the respiratory cycle, to fish in which the column of maximum occurrence lies closer to the start of the previous respiratory cycle in columns 7, 8 or 9. Experimental evidence to be presented in the next section suggests that a vagally mediated reflex is concerned in this co-ordination. A cyclically fluctuating pattern of cardiac inhibition, related to dilatation of the pharynx at each respiration, is superimposed on a tonic background of vagal activity related to blood pressure. Thus any lowering of this tonic activity caused by blood loss or injury might be expected to permit the heart to beat at a phase of the respiratory cycle closer in time to the cyclic peaks of inhibition resulting from respiration. Since this was just the type of deviation seen and as it tended to occur in fish with injuries sustained during their capture, it has been regarded as atypical.

A corollary of this co-ordination was that the time elapsing between one heart beat and the next, the E.C.G. interval, approximated to some whole multiple of respiratory cycle length. In fish showing the alternation of longer and shorter cardiac cycles (Fig. 2B) a histogram of cardiac cycle length showed a markedly bimodal distribution.

The mechanism of co-ordination

The co-ordination outlined in the previous section could be effected in various ways. The following three must be considered.

(1) If the heart were sensitive to stretch, the influx of venous blood resulting from the compression of the anterior cardinal sinuses during pharyngeal contraction might cause the heart to beat. The tendency for the heart to beat just after pharyngeal contraction has already been noted, and the failing dogfish heart can be made to beat by dilating it with Ringer's solution.

(2) The respiratory and cardio-inhibitory centres are anatomically closely related in the vertebrate medulla; it is possible that there might be some irradiation of

influences from one to the other. Rhythmical bursts of neuronal activity in respiratory centres might also activate the cardio-inhibitory centre through purely central pathways. Such explanations have been advanced to account for sinus arrhythmia in mammals (Heymans, 1929).

(3) The heart might be reflexly inhibited, the efferent pathway being the cardiac vagi. The afferent pathway could consist of receptors fired by pharyngeal dilatation and relaying their impulses to the medulla in the branchial nerves.

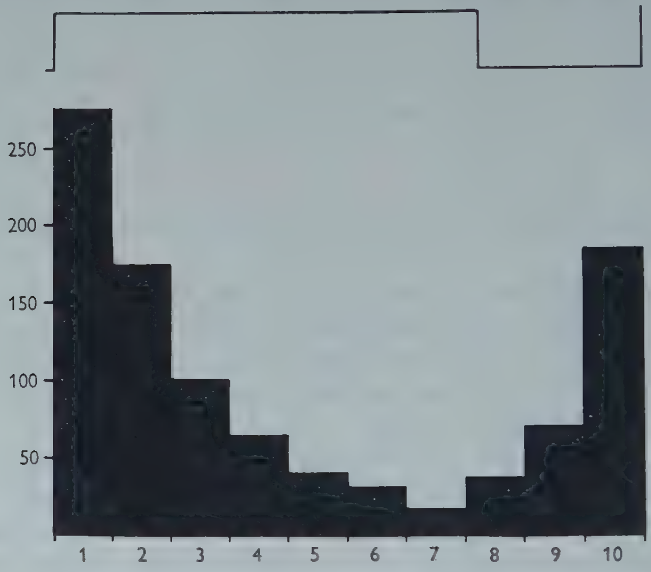

Fig. 3. The occurrence, in the ten phases of a respiratory cycle, of 1000 heart beats recorded as ten runs of 100 each, from ten specimens of *S. lebruni* between 4 and 16° C. Above, a respiratory cycle, with mouth opening indicated as an upstroke.

Artificial inflation of the pharynx with a small balloon provided evidence on this question. If such inflations were small enough, it was possible to avoid provoking the cough reflex and to show that they evoked respiratory inhibition and cardiac slowing (Fig. 2C). The respiratory inhibition has been described in a previous paper (Satchell, 1958); it is believed to be evoked by the activity of receptors fired by pharyngeal movement. When the anterior cardinal sinuses of both sides were opened and blood flow through them prevented by plugging them anteriorly and posteriorly, neither co-ordination nor the response to inflation was disrupted. But cutting the cardiac branches of the vagus caused the heart to accelerate from 12 to 18 beats per min., whilst co-ordination disappeared and the cardiac, but not the respiratory, response to inflation was lost. These findings are opposed to a haemodynamic explanation since a mechanism dependent on blood flow ought to be affected by plugging the anterior cardinal sinuses but ought not to be disrupted by cutting the cardiac vagi.

The action of atropine is also opposed to a haemodynamic explanation. Intravenous atropine 0·2 mg./kg. produced the same effects as cutting the cardiac vagi;

co-ordination and the cardiac response to inflation disappeared and the heart accelerated. Atropine did not cause further cardiac acceleration in the vagotomized fish, indicating that its cardiac effects were exerted on the vagal terminals rather than on the heart itself. The respiratory response to inflation persisted, suggesting that it does not involve an atropine-sensitive cholinergic link. Likewise, destruction of the medulla caused the heart to accelerate and the response to inflation to disappear. These findings implicate the cardiac vagus and its cholinergic link with the heart in this co-ordination; they do not distinguish between the second and third explanations.

That the co-ordination was not effected through purely central pathways, but involved a peripheral link, was suggested by the action of curare. When the respiratory muscles were paralysed the characteristic variation from beat to beat in cardiac cycle length was lost. Records of nerve action-potentials in the branchial branches of the vagus demonstrated that the respiratory centre continued to send out its volleys for many hours after curarization, if a stream of water was directed through the spiracle. That curare was not blocking the central and efferent pathway was shown by the ease with which respiratory and cardiac inhibition could still be produced when the pharynx was artificially inflated. But the absence of any spontaneous movement would deprive a cardiac inhibitory reflex of its afferent component and thus prevent co-ordination.

The following facts may be adduced in favour of the reflex explanation. An efferent limb, the cardiac vagi, has already been established in the process of excluding the other hypotheses. In a previous paper (Satchell, 1958, Fig. 3) it has been shown that bursts of action potentials could be recorded in the distal end of a cut branchial nerve of *S. lebruni* synchronous with each pharyngeal contraction. Inflation of a balloon in the pharynx produced a discharge containing a phasic element at inflation and deflation, and a sustained response throughout the period of inflation. Some of these fibres and the receptors connected to them could well constitute the afferent limb of the reflex. The presence of a system of reflexly linked cardio-inhibitory fibres in the branchial nerves has been established by tetanically stimulating the central end of a cut branchial nerve (Fig. 2D). Cardiac inhibition was always produced, as has been shown in the eel by Mott (1951) and in the dogfish by Lutz (1930*b*).

Significance of the branchio-cardiac reflex

It is possible that a reflex that links the activity of two muscle pumps, one propelling blood through a system of sinuses, and the other water over them, might enhance diffusion by synchronization of the periods of rapid flow. With this hypothesis in mind, attempts have been made to determine the time course of the pressure differential across the gill vessels. The input pressure to the gill capillaries has been sampled by cannulating the first afferent branchial artery, the pressure beyond the gills by cannulating the coeliac artery so that the cannula tip lay in the dorsal aorta within 3 cm. of the point of entrance of the epibranchial arteries. The type of manometers and their connexions have already been described. As this is the first time that fish blood-pressure traces have been recorded with modern electrical manometers having a high frequency response some general comments may be made.

The pressures recorded were as follows, in mm. Hg.

	Systolic pressure	Diastolic pressure
1st branchial artery	22·8	15·4
Anterior dorsal aorta	17	13·3

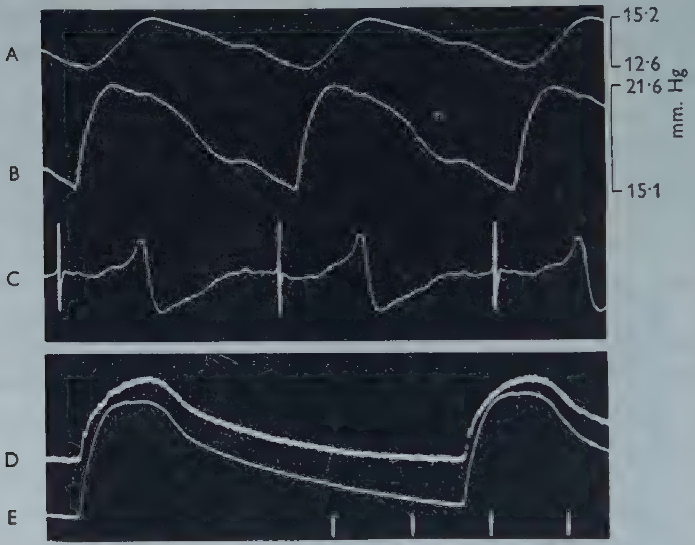

Fig. 4. Oscilloscope tracings all from one specimen of *S. lebruni*, 14° C. A and B recorded simultaneously. C. Placed in appropriate time relation. D and E recorded simultaneously. A and B: blood pressure from coeliac artery and 1st afferent branchial artery, respectively. *n* = dicrotic notch; calibration on right. C. E.C.G. The *QRS* stroke has been retouched. D. Gill opacity recorded with phototransistor. E. Afferent branchial artery blood pressure. D and E after curarization. Time = sec.

These pressures are within the range of those recorded by other workers from fish of comparable size ($4\frac{1}{2}$ lb.) (see Brown, 1957, for references). They are probably lower than normal, due to the trauma resulting from the double cannulation; in fish in which only the branchial artery was cannulated systolic pressures as high as 33 mm. Hg were recorded.

The afferent branchial artery trace shows a steeply rising front, following the ventricular contraction, and a more gentle decline. The dicrotic notch, as the semilunar valves closed, is just visible (Fig. 4B, *n*), and the valves thus stayed open for 1·55 sec. at a heart rate of 22/min. The closure of the valves occurs after the *T* wave of the E.C.G., suggesting that, as in mammals, it is the relaxation of the heart muscle, leading to a decline in intraventricular pressure and a backward flow of blood, that initiates closure. The small peaks of blood pressure before and after the dicrotic notch were observed to be caused by expiration.

The trace from the dorsal aorta shows the expected lower pulse pressure, and the delay in the onset of the rise following systole and expiration.

As the circulatory system of fish has the smaller vascular bed of the gills in series with the larger vascular bed of the body, enough blood must flow through the gill vessels, early in each cardiac cycle, to distend the large elastic reservoir of the peripheral circulation. As this occurs, pressures should rise in the dorsal aorta, the pressure differential across the gills should fall and the rate of flow through them should slacken. Thus immediately following ventricular systole there should be a brief period of rapid flow through the gills. To test this hypothesis ten successive branchial artery and aortic pressure traces have been projected on to graph paper, and the pressure values, at twenty-three successive points, determined and averaged. From these the two curves A and B in Fig. 5 have been constructed. By subtraction, curve C has been derived, representing the pressure difference across the gills. It can be seen that this curve reached a maximum of 8 mm. Hg before the peak of the afferent artery pressure curve, and that after the semilunar valves had closed (12th–14th points on the graph) the curve of pressure difference changed slope and declined more slowly. At this stage the pressure difference across the gills was only some 2½ mm. Hg and gradually decreased as the elastic reservoir of the ventral aorta poured its contents into the emptying reservoir of the peripheral circulation.

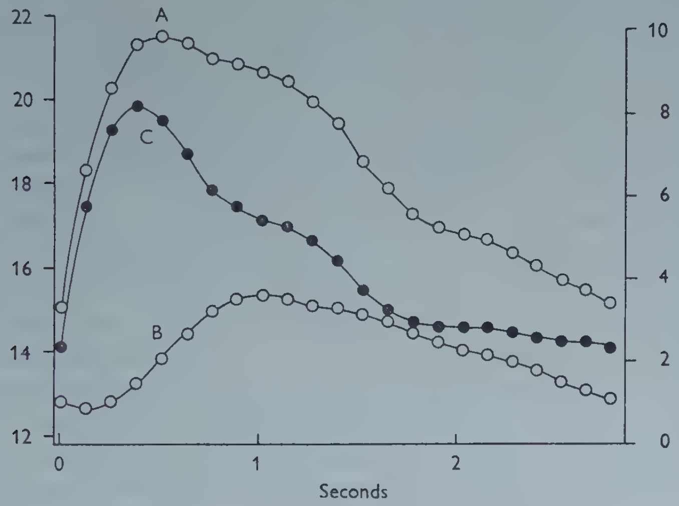

Fig. 5. *S. lebruni*, 14° C. Blood-pressure records from A, the 1st afferent branchial artery, and B, the coeliac artery, derived by averaging ten simultaneously recorded consecutive pairs of traces. C. Differential pressure across the gill circulation, derived by subtracting B from A. Ordinate for A and B on left, for C on right, in mm. Hg.

Changes in pressure across the gills would lead to comparable changes in flow only if the resistance of the branchial vessels remained constant. Cyclic activity in a system of branchial vasoconstrictor fibres could minimize the fluctuations in flow. The evidence of previous workers is against the existence of such a system (Wyman & Lutz, 1932), but to gain further evidence on this the opacity of the gill was monitored with a red-sensitive phototransistor and light source. The device proved to be remarkably sensitive to changes in the amount of blood in the gill and Fig. 4 D and E show the simultaneous recording of gill opacity and afferent branchial arterial blood pressure in a curarized dogfish. The opacity curve followed the pressure curve so closely as to suggest strongly that the vessels must be compliant, increasing in diameter as the pressure rose and narrowing as it fell. In some experiments, but not in the one depicted in Fig. 4, even the small excursion of the dicrotic notch produced a corresponding change in the opacity. Whilst there is thus no evidence of active vasoconstriction of branchial vessels, their passive dilatation might be expected to affect the pressure-flow relationships. The equations concerning the flow of blood through a compliant tube have not yet been completely solved (Rashevsky, 1945; Branson, 1945), but as flow is proportional to the 4th power of the radius (Poiseuille's Law) the early peak of pressure difference across the gill must result in a greater increase of flow than a linear pressure/flow relationship would predict.

It remains to demonstrate that this early period of rapid blood flow through the gills coincides with the phase of rapid water flow past them. In the experiment depicted in Fig. 4, the simultaneous recording of the two blood pressures was followed by the recording (also simultaneous) of the afferent branchial pressure and the electrocardiogram. It has thus been possible to calculate that the time elapsing between the occurrence of the *P* wave of the E.C.G. and the start of the pressure rise was 0·690 sec. (S.D. = 0·0146 sec.) and from the data used in the construction of Fig. 5 the time from the start of the pressure rise to the peak of the differential pressure was determined as 0·251 sec. Adding these, it can be said that under the conditions of this experiment ($T = 14^\circ$ C.) 0·94 sec. elapsed between the start of a heart beat and the peak of pressure difference across the gills. The respiratory rate in ten unanaesthetized fish recorded at temperatures of 14–16° C. was 54·2 (S.D. = 3·79), so each tenth of a respiratory cycle endured for 0·11 sec. If, for convenience, all heart beats originating in phase 1 (the phase of maximum occurrence) were regarded as starting at the same instant half-way through the phase (0·05 sec. from the start), the lapse of 0·94 sec. would bring the time of peak blood-pressure difference across the gills to 0·99 sec. after mouth opening. With each phase of respiration enduring for 0·11 sec., the peak of blood pressure difference would occur at the division between the 9th and 10th phases ($9 \times 0{\cdot}11 = 0{\cdot}99$) of the respiratory cycle. Reference to Fig. 1 B shows that this is indeed the time when water pressure in the pharynx was maximal. The evidence thus indicates that heart beats with the *P* waves starting immediately after mouth opening will result in a peak of blood flow through the gills coincident with the maximum flow of water over them.

DISCUSSION

It is now reasonably established that in fish as in mammals blood pressure is reciprocally related to heart rate. In the eel (Mott, 1951) and the dogfish (Lutz & Wyman, 1932) raising the blood pressure slows the heart. The structure and location of the pressor receptors in the branchial vessels of dogfish have been studied by Boyd (1936); bursts of action potentials in branchial afferent fibres, synchronous with cardiac systole and evoked by increasing blood pressure, have been demonstrated by Irving, Solandt & Solandt (1935) in dogfish. Cardiac inhibition evoked by electrical stimulation of the central end of a cut branchial nerve has been described by Mott (1951) in the eel, and by Lutz (1930*b*) in the dogfish. We may thus safely conclude that there is a reflex control of heart rate, the number of respirations intervening between one heart beat and the next depending on the level of tonic vagal activity. The cyclic inhibition reflexly induced by respiratory movement is superimposed on this, controlling the position within a given respiratory cycle at which an incipient beat will occur. Whether receptors distinct from those subserving the blood-pressure reflex are concerned in the branchiocardiac reflex is unknown.

Hughes & Shelton (1958) have shown that in teleosts the opercular suction pump alternates with the buccal pressure pump to maintain a flow of water across the gill throughout almost all of the respiratory cycle. There is no operculum in recent elasmobranchs, but Woskoboinikoff (1932) has suggested that the outer portion of each gill pouch acts as a suction pump, and that the two pumps act synchronously. Water flow across the gills is thus rapid during approximately one-third of the respiratory cycle and less rapid during the remainder. Were the period of rapid blood flow to occur when the water flow was minimal, it might be that partly deoxygenated blood would be poured into the peripheral circulation. The branchiocardiac reflex, by causing the periods of peak flow to coincide, ensures a maximum gradient of oxygen tension across the gill epithelium, and hence the most effective oxygenation of the blood. Van Dam (1938, quoted in Krogh, 1941) has shown that, in fish, water and blood flow on a counter-current pattern. This results in the most highly oxygenated blood being partnered with the most highly oxygenated water, so that the greatest gradient of oxygen tension is maintained along the length of the vessel. The branchiocardiac reflex may be regarded as achieving a temporal coincidence of flow rates analogous to the spatial coincidence of oxygen tensions secured by the counter-current arrangement of the gill circulation.

SUMMARY

1. The hearts of *Squalus lebruni* and *Mustelus antarcticus* beat at intervals that approximate to some simple multiple (1–4) of respiratory cycle length.

2. The beat tends to be initiated during a particular phase of the respiratory cycle, most commonly just before or just after the opening of the mouth.

3. Possible mechanisms of this co-ordination are considered and evidence is presented to show that it is reflexly mediated. Fibres from receptors fired by pharyngeal dilatation are believed to constitute the afferent limb, and the cardiac vagi the efferent limb of the reflex arc.

4. Simultaneous records of blood pressure have been made from the 1st afferent branchial artery and the coeliac artery. By subtraction, the differential pressure across the gill vessels has been calculated and found to have a peak early in the cardiac cycle.

5. Simultaneous photo-electric records of gill opacity and branchial blood pressure show the gill vessels to be compliant. It is concluded that there is a brief period of rapid blood flow early in each cardiac cycle.

6. Calculations from simultaneous records of the electrocardiogram and branchial blood pressure showed that heart beats originating near the time of mouth opening caused a rapid flow of blood through the gills at the time that water was expelled across them.

7. It is suggested that this synchronization of the periods of rapid flow of blood and water serves to maintain the diffusion gradient of oxygen across the gill epithelium.

It is a pleasure to acknowledge my indebtedness to Mr Vic. Hansen who has supplied me with so many excellent fish; to Profs. A. K. McIntyre and J. R. Robinson, who have read the manuscript and to Mr A. Annand who designed and made much of the electronic equipment. Thanks are also due to the Medical Research Council of New Zealand who supported the project.

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Note added in proof

The paper 'The mechanism of gill ventilation in the dogfish and the skate', by G. M. Hughes (*J. Exp. Biol.* **37**, 11–27, 1960) did not arrive in New Zealand in time to be considered in this study. Hughes divides the respiratory cycle into four phases. Mouth opening (the start of the respiratory cycle in this paper) occurs half way through phase 4; mouth closing occurs at the end of phase 2. Hughes' Fig. 4 (p. 18) suggests that heart beats originating in column I (the time of maximum occurrence) would result in peak blood flow through the gill vessels during the operation of the buccal pressure pump. Beats originating in column 7 (the column of minimum occurrence) would result in peak flow coinciding with Hughes' phase 2 when water pressure accross the gills is minimal.

THE EFFECTS OF IONS AND DRUGS ON NEUROMUSCULAR PREPARATIONS OF SEA ANEMONES

I. ON PREPARATIONS OF THE COLUMN OF *CALLIACTIS* AND *METRIDIUM*

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INTRODUCTION

Following Pantin's (1935) demonstration of facilitation in the quick closing response of the sea anemone, *Calliactis parasitica*, the effects of ions and drugs on the facilitated response in this animal, and in *Metridium senile*, were studied in detail (Ross & Pantin, 1940; Ross, 1945, 1952). There was evidence that some ions had specific effects on facilitation at the neuromuscular junctions but, as the work was done on whole animals, some of the effects observed might have arisen at other places. Moreover, many drugs had no effect; it was possible that they failed to reach the neuromuscular junctions.

Techniques have now been developed for the study of isolated preparations of anemones (Batham & Pantin, 1954; Ross, 1957*a*), which offer more favourable material for investigating the effects of such treatments at definite sites of action and enable substances to reach the neuromuscular junctions more easily. In these preparations effects on the muscles which give quick responses can be distinguished from effects on muscles involved in slow movements. The earlier work gave almost no information about the action of ions and drugs on slow activities and responses, which have many interesting features of their own. For these reasons, it was desirable to extend the study of ion and drug effects to isolated preparations of *Calliactis* and *Metridium*.

A preliminary report has been published on the effects of tryptamine and 5-hydroxytryptamine on both the quick and slow types of preparations (Ross, 1957*b*). Of the present papers, the first deals with preparations giving only slow responses to stimuli and showing only slow spontaneous activities, viz. preparations taken from the columns of these animals. The second will deal with work on preparations which give both quick and slow responses to stimuli, and in which inherent activity is weak or non-existent, viz. the marginal sphincters of *Calliactis* and *Metridium* (Ross, 1960).

METHODS

Preparations were made by cutting rings of *Calliactis* and *Metridium* after several hours anaesthesia in a mixture of equal parts sea water and $MgCl_2$ (0·4 M) (Batham

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& Pantin, 1954; Ross, 1957*a*). After about 2 days' recovery in sea water the rings were set up in a muscle bath for recording on a slowly rotating kymograph. Using a Perspex electrode-hook (Ross, 1957*a*), responses to stimuli could be recorded as well as the normal activity of the preparation. Stimuli came from a condenser discharged at appropriate frequencies (intervals between shocks ranging from 1 to 10 sec.) and reached the preparation through platinum tips on the electrode-hook.

For some experiments ring preparations with a strip of column attached were used to differentiate between the effects of treatments applied directly on or near the contractile tissue, and effects on the conducting system and on receptors at some distance from the muscle. This was done in a shallow bath with a paraffin wax inset across which ran a slightly raised barrier. There was a difference in level of about 5 mm. in the wax on either side of the barrier. On the low side, the strip of column was pinned down and immersed in fluid. On the upper side beyond the barrier, the preparation was fixed where the loop joined the strip. The other end of the loop, attached to the writing lever of the kymograph, was suspended in air and irrigated continuously by fluid from an overhead supply. This fluid ran away on the far side of the wax inset without meeting or mixing with the fluid immersing the strip of column, which could also be changed independently. A short piece of column at the barrier was unavoidably out of contact with either fluid and had to be kept moist by flooding occasionally with sea water. Although this arrangement was not ideal, it enabled the contractile and non-contractile parts of the strip preparations to be tested independently as desired.

NORMAL PROPERTIES OF THE COLUMN PREPARATIONS

Isolated ring preparations of the column of *Metridium* or *Calliactis* show intermittent slow contractions and relaxations. Batham & Pantin (1954) described this activity in *Metridium*, and also the responses to electrical stimuli in preparations with longitudinal strips of column attached. Needler & Ross (1958) showed that column preparations from *Calliactis* have similar properties. While this work has been in progress, Ewer (1960) has been investigating the activities of preparations from the column of *Calliactis* and has shown up differences in activity and responses at different levels of the column and in preparations of different ages after the operations.

Compared with most smooth muscle preparations from isolated organs, these isolated rings of the column show a good deal of variety in their activities. The following is a general description of the mechanical record, examples of which are seen in the figures accompanying this paper. A preparation attached to a lever under load soon takes up a stable basic length. Periodically, and quite suddenly, a contraction begins which, under light load, may close the ring completely. Equally suddenly, without the maintenance of tension, relaxation sets in and brings the preparation back to the basic length. The rate of relaxation decreases as the basic length is approached, and except for the occasional occurrence of small contractions this basic tonus is maintained for some time before the next powerful contraction begins. Over long periods, in the absence of stimulation, the record shows an irregular rhythm of big movements every 10–20 min., each one lasting 2–3 min., alternating with much longer periods of relaxation interrupted by a highly variable number of small movements.

Electrical stimuli, delivered when the preparation is extended, evoke responses which reproduce many features of the spontaneous movements. Ten to twenty stimuli separated by intervals of between 1 and 10 sec. will usually elicit a maximal contraction. Fewer stimuli produce submaximal contractions and as a rule three or four stimuli are required to cause any shortening at all.

An outstanding feature of these responses to stimuli, as Batham & Pantin (1954) pointed out, is their long latency, usually not less than 30 sec. and often as long as 2 min. or more. In these column preparations the latency of the response varies with frequency and number of stimuli, as does the slow response of *Calliactis* (Ross, 1957*a*). Table 1 gives latent periods of responses to five stimuli at four frequencies in eight different preparations. At 1 stimulus per sec. the mean latencies of responses to 2, 3, 4 and 5 stimuli were respectively: 32, 28, 27 and 21 sec.

Table 1. Metridium *column preparations. Latent periods of responses to five stimuli at four frequencies*

		Latent periods of response to five stimuli (sec.)			
Frequency (intervals between stimuli, sec.) ...		1·0	4·9	9·0	14·5
Preparation	M1	30	75	90	110
	M5	37	50	73	90
	M6	37	55	80	120
	M7	30	45	32	45
	M9	12	19	28	47
	M10	15	25	27	62
	M11	26	55	65	95
	M14	15	27	30	40
	Means	25·2	48·7	53·1	76·1

The latency of the response of a given preparation to standard groups of stimuli is consistent over periods of time up to many hours. However, when preparations are studied over several days (this is possible as good preparations may survive for more than 10 days) changes in excitability and latency occur. Ewer (1960) has shown that the latent period usually gets shorter with the passage of time. He attributes this to a decline of an inhibitory condition which is particularly evident in freshly prepared rings from the pedal edge or lower part of the column. Rings including the pedal edge have not been used in this work and most of the experiments have been done on rings from the submarginal and mid-column levels, where changes in excitability are less conspicuous.

These preparations are also sensitive to stretch, to reduction of load and to general tactile stimulation; all of these cause vigorous contractions. The latency of responses to strong tactile stimuli or reduced load is of the same order of magnitude as that of responses to electrical stimuli. The contractions elicited by stretching the preparations, however, have much shorter latent periods, as the following figures show. In five preparations, each tested five times, the mean latent periods of responses were: to ten electrical stimuli (1·1 sec. between stimuli)–36 sec.; to strong tactile stimulation—30 sec.; to unloading—30 sec.; to stretch—10 sec. This difference may be important in determining the relation between responses to stimuli and normal inherent activity.

Given preparations which are so variable in their inherent activity and their responses, the effects of treatments can only be determined for each preparation after a long period of preliminary recording and testing of responses. Data on the following are considered to be significant in estimating the effects of any treatment applied: (1) 'direct' contractions (see below); (2) the tonus, or basic length, of the preparation when fully relaxed between contractions; (3) the frequency and magnitude of the contractions in the inherent activity; (4) the size, and (5) the latency of the response to a given number of electrical stimuli at a given frequency.

THE ACTION OF IONS

The effects of altering the ionic environment of the column preparations were studied in two ways: (1) adding measured amounts of isotonic solutions of KCl, $CaCl_2$ and $MgCl_2$ to natural sea water; (2) treating preparations with artificial sea waters lacking various constituent ions using the data compiled by Pantin (1946). The results are summarized in Tables 2 and 3, which deal with single and multiple ion effects, respectively.

In these tables, and in Tables 4–6 dealing with drug effects, an attempt has been made to express the results quantitatively, even though the standards for such an expression are very inexact and arbitrary. The '0', '+' and '−' ratings used in the tables are based on the following scheme. A treatment was presumed to have no effect if no change of any kind was observed in the course of four preliminary tests. If any effects, or possible effects, were observed in the preliminary tests, four more tests were carried out, or more if it were necessary to decide whether an effect occurred or not. Most of the results were based, therefore, on eight tests. Treatments were usually given when the preparation was relaxing after one of its normal spontaneous contractions, when another movement was not to be expected for perhaps at least another 10 min. A contraction was presumed to be a 'direct contraction' in response to the treatment if it occurred within 5 min. of the introduction of the solution or the drug. If such contractions were observed in only 2–3 of the tests, it was rated '+'; if in 4–5 of the tests, it was rated '++', and if in 6 or more of the tests, it was rated '+++'. Similarly, a rise of tonus amounting to 25–50% of the full contraction was rated '+', a rise of 50–75% was rated '++' and a rise of 75% or more was rated '+++'. The same arbitrary gradings, 25–50%, 50–75%, and 75% and over were used to provide '+', '++' and '+++' ratings for comparing spontaneous activity and responses to stimuli before and during treatments. Where negative effects were evident, the same grades were used to provide '−', '−−' and '−−−' ratings for depressant treatment.

SINGLE ION EFFECTS

Potassium

Exposing preparations to excess K+ affects both tonus and activity. The change in tonus takes the form of an apparent direct contraction, or series of contractions, which usually occur within 1–2 min. With such an early response it is not difficult to distinguish between direct contractions and the spontaneous contractions which can occur at any time, although they are usually at least 10 min. apart.

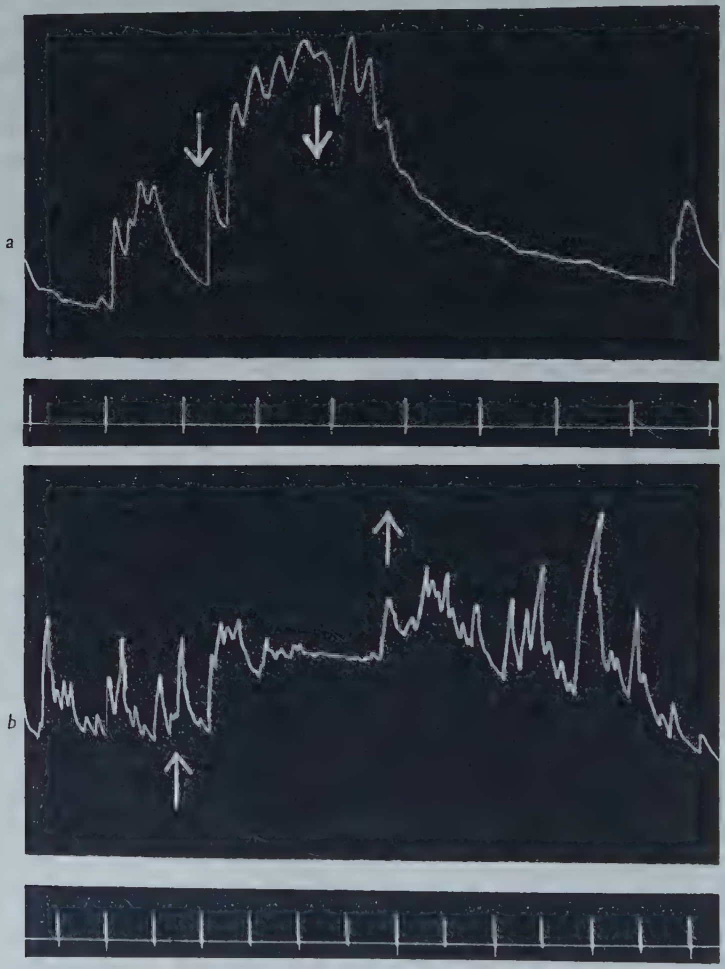

Fig. 1. (*a*) *Metridium* column preparation treated with K+ × 8 for 10 min. Time trace, 6 min. (*b*) *Calliactis* column preparation ('low column') treated with K+-free sea water for 60 min. Time trace, 15 min.

Fig. 1 *a* shows the effect of increasing K+ × 8 on a ring of the column of *Metridium*. The direct contraction is always followed by a maintained rise in tonus and an increased frequency of beat at the new level. All these effects are more pronounced the greater the excess of K^+. Effects on *Calliactis* are similar. This type of activity continues for at least an hour (longer exposures were not tested) without showing any depressant effects (cf. Mayer, 1906). Recovery from excess K^+ usually takes the form seen in Fig. 1 *a*, viz. steady relaxation without spontaneous activity.

The size and latency of the responses to stimuli after exposure to excess K^+ cannot be compared with those of the controls. This is because there is so much more movement and the preparations are almost fully contracted at the new level of tonus. When K^+ ions are only slightly increased (×2) and the direct effects are small, no significant changes in the size and latency of the responses are apparent.

The effects of K^+-free sea water are difficult to assess. Immediate effects are slight, but after about ½ hr. activity begins to decline or becomes erratic. Effects on tone are not consistent and the diminished or erratic activity may be associated with a moderate rise in tonus, as in Fig. 1 *b*, or with a relaxed condition. Even after prolonged exposure to K^+-free sea water preparations still respond to stimuli. On return to natural sea water normal activity begins almost at once (Fig. 1 *b*). Potassium is, therefore, not essential for the responsiveness of the column musculature, though in its absence normal activity cannot continue indefinitely.

Table 2. *Effects of single ion excess or defect on column preparations of* Calliactis

		Effects of treatments			
		Direct contractions	On tonus	On activity	On responses
Excess K^+	×2	+	+	o	o
	×4	++	++	o	+
	×8	+++	+++	+	+
Excess Ca^{2+}	×2	o	o	o	o
	×4	o	o	o	+
	×8	o	+	+	+
Excess Mg^{2+}	×2	o	o	− −	− −
	×4	o	−	→ Ab	→ Ab
K^+-free		o	o	→ Ab	o
Ca^{2+}-free		o	−	→ Ab	→ Ab
Mg^{2+}-free		+++	+++	+++	Unobs.
Na^+-free		$o^{s}/+^{c}$	$-^{s}/+^{c}$	$-^{s}/+^{c}$	o

(For explanation of use of + and − notation see text. No effect recorded as 'o'. → Ab indicates depression of activity or responses leading to abolition; 'Unobs.' = unobservable; s, NaCl replaced by sucrose: c, NaCl replaced by choline chloride.)

Calcium

The effects of excess Ca^{2+} are problematical. In at least half my experiments, increasing Ca^{2+} up to ×8 had no detectable effects. Possible direct contractions are almost non-existent, but in some cases increased activity and incomplete relaxation produced a gentle rise in tone (Fig. 2 *a*). In some cases, also, responses to stimuli are enhanced (Fig. 2 *b*). Although the actions shown in the illustrated examples might rank as major effects, the large proportion of negative results gives excess Ca^{2+} the overall low rating ascribed to it in Table 1, since inconsistency of action may be regarded as a sign of low activity.

In the absence of Ca^{2+} the activity and the responsiveness of the column prepara-

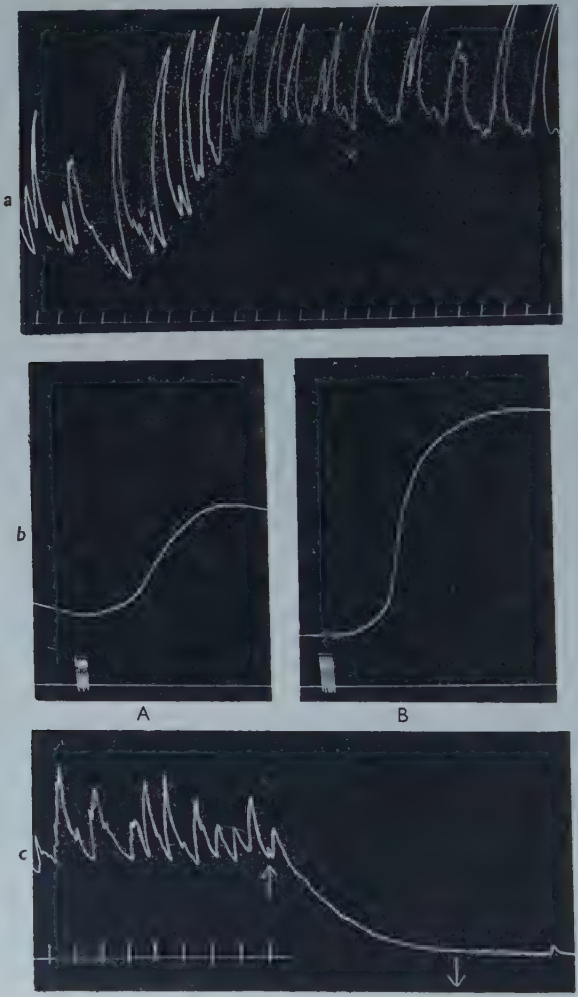

Fig. 2. (*a*) *Calliactis* column preparation ('subsphincter') treated with $Ca^{2+} \times 8$ for 57 min. Time trace, 6 min. (*b*) Responses of *Calliactis* column preparation ('low column') to five stimuli at 1 in 1·1 sec.: A, in natural sea water before treatment (latency 8 sec.); B, in sea water $Ca^{2+} \times 4$, 92 min. after introducing $CaCl_2$ (latency 6 sec.). (*c*) *Calliactis* column preparation ('mid-column') treated with Ca^{2+}-free sea water for 32 min. Time trace, 6 min.

tions soon disappear. Fig. 2*c* shows an example of this effect. During this treatment the preparations relax steadily; they do not remain inactive while holding the original basic length. Responses to stimuli in Ca^{2+}-free sea water are at first much smaller and have a longer latency while, after about 10 min., the preparations become quite inexcitable. Unlike K^+, Ca^{2+} is therefore essential for the inherent activity and excitability of the column musculature.

Magnesium

As $MgCl_2$ is generally used as an anaesthetic in these animals, a rapid depressant action on the column preparations was to be expected. Fig. 3*a* shows such an effect with $Mg^{2+} \times 4$. Movement ceases within 2–3 min. and a period of slow relaxation sets in during which the preparation is quite inexcitable. Less drastic excesses of Mg^{2+} produce the same effect more slowly, and it was possible to show in such tests that Mg^{2+} also depresses the size and increases the latency of the response to electrical stimulation before the preparation becomes inexcitable.

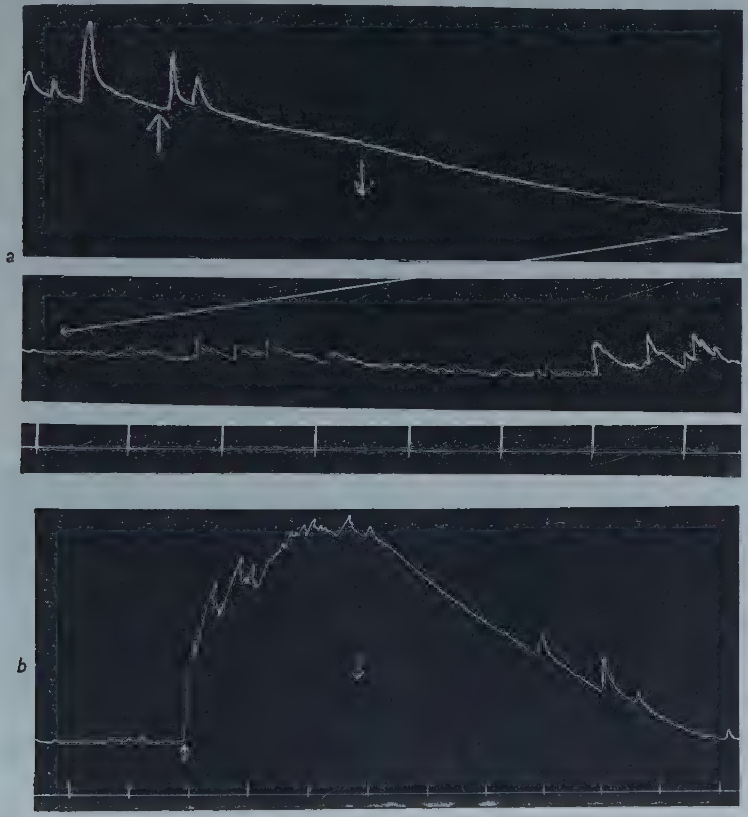

Fig. 3. (*a*) *Metridium* column preparation treated with $Mg^{2+} \times 4$ for 32 min. (lower trace follows directly from upper trace). Time trace, 15 min. (*b*) *Calliactis* column preparation (mid-column) treated with Mg^{2+}-free sea water for 30 min. Time trace, 10 min.

Mg^{2+}-free sea water produces one of the most striking effects observed on these preparations. A series of contractions in quick succession shortens the preparation almost to its full extent and then contractions and relaxations follow each other very rapidly as in a partial tetanus. Fig. 3*b* shows this phenomenon with contractions occurring at a frequency of about 1 per 1–2 min. Recovery takes the form of a rapid return to the original basic length and renewal of the original slower level of activity. As with excess K^+, it was impossible to carry out any tests on responses to stimuli in Mg^{2+} free sea water because of the greatly increased tonus and intense activity of the preparations.

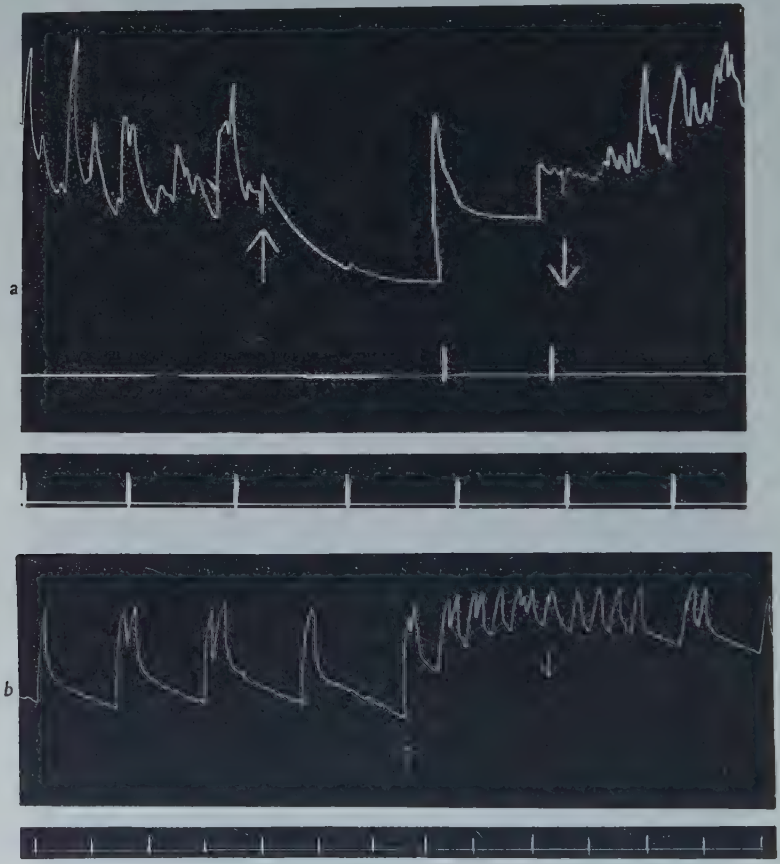

Fig. 4. (*a*) *Calliactis* column preparation ('subsphincter') treated with Na^+-free (sucrose substitution) sea water for 38 min. Stimulated by ten shocks at 1 in 1·1 sec. at times indicated by signals (22 and 36 min.). Time trace, 15 min. (*b*) *Calliactis* column preparation ('mid-column') treated with Na^+-free sea water (choline chloride substitution) for 40 min. Time trace, 15 min.

Sodium

Replacing the sodium chloride of sea water by sucrose has a prompt depressant effect on the activity of the preparation but leaves the responses to electrical stimulation virtually unimpaired (Fig. 4*a*) at least for exposures up to 30 min. If the sodium chloride is replaced by choline chloride, in order to maintain Cl ions at their normal concentration, the result is different. An early direct contraction followed by enhanced activity at a raised level of tonus is the usual effect of the treatment (Fig. 4*b*). Large doses of choline alone (4×10^{-3}) also cause direct contractions but do not raise tonus or effect activity to any extent. The enhanced activity when NaCl is replaced by choline chloride shows that absence of Na alone does not seriously impair the working of the column musculature; it would seem that the depression seen in Fig. 4*a* is due to Cl^- lack and not to the absence of Na^+.

Other ions

Column preparations of *Metridium* and *Calliactis* soon shorten when exposed to sea water of pH 5 or 4, but after these initial contractions, activity continues at the shorter length without any marked increase in frequency. Recovery was generally slow. Alkaline sea water at pH 10 had similar effects.

Artificial sea waters lacking sulphate and bicarbonate ions were also tested but no significant effects on tonus or activity were observed.

In review, the pattern of single ion effects is as follows. With K^+ and Ca^{2+}, excess and deficiency do not have simple opposing effects. Excess K^+ is highly excitatory but K^+-free sea water is only moderately inhibitory in its effects. Excess Ca^{2+} is only slightly excitatory whereas Ca^{2+}-free sea water is completely inhibitory. Only Mg^{2+} shows simple opposing effects with excess and deficiency, the former being highly inhibitory and the latter causing a spectacular hyperactivity. The ionic requirements for normal working seem to be that K^+ should not *exceed*, that Ca^{2+} should not *fall below* and that Mg^{2+} should *neither exceed nor fall below*, their concentrations in sea water. Obviously Mg^{2+} is particularly important in providing the ionic conditions for normal excitation and neuromuscular activity.

MULTIPLE ION EFFECTS

The results of tests on the effects of excess or lack of two or more cations simultaneously are set out in Table 3 and illustrated in Fig. 5. The effects of such treatments were never more profound than those produced by excess or lack of particular single ions. The special significance of Mg^{2+} is indicated by the fact that the effects of Mg^{2+}-excess and Mg^{2+}-lack cannot be offset by simultaneously raising or lowering Ca^{2+} or K^+ concentrations. The hyperactivity associated with Mg^{2+}-free sea water is only abolished when both Ca^{2+} and K^+ are removed, i.e. in NaCl only. The effect of NaCl only, i.e. without Ca^{2+}, K^+, Mg^{2+}, SO_4^{2-}, HCO_3^-, Br^-, is interesting. After an initial direct response, tone is usually maintained at a higher level with activity continuing for about 15–30 min. and then gradually coming to a standstill (Fig. 5*d*). Recovery afterwards is rapid compared to that following excess Mg^{2+} or Ca^{2+}-free sea water. The NaCl effect as a whole is slower and less specific than the single ion effects described above.

It is noteworthy that Ca^{2+}-free sea water only stops all activity when Mg ions are present; sea water lacking both Ca and Mg acts exactly like Mg^{2+}-free sea water alone (Fig. 5*a*). Partial restoration of Mg^{2+} in such circumstances produces the inhibition associated with Ca^{2+}-free conditions (Fig. 5*b*). This suggests that the Ca^{2+}-free inhibition is due to the great excess of Mg^{2+} over Ca^{2+}, and perhaps only another form of the inhibitory effect of Mg^{2+} excess.

It appears that K^+, Ca^{2+} and Mg^{2+} have specific functional roles in maintaining the balanced conditions for the sequence of contractions and relaxations that make up the normal activity of these muscles. These roles might be summarized as follows: K^+ is excitatory but not essential for contraction; Ca^{2+} is not excitatory or inhibitory but is essential for contraction in the presence of Mg^{2+}; Mg^{2+} is essential for relaxation in the presence of K^+ and Ca^{2+}.

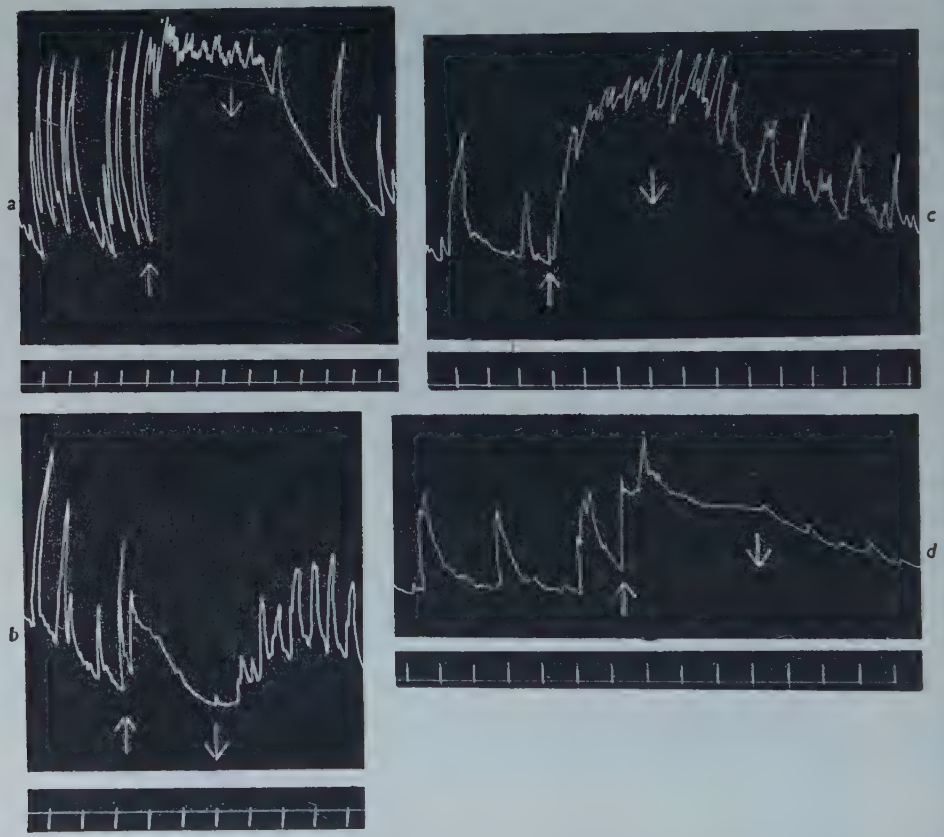

Fig. 5. (*a*) *Calliactis* column preparation ('subsphincter') treated with sea water lacking both Mg^{2+} and Ca^{2+} for 40 min. (*b*) *Calliactis* column preparation ('mid-column') treated with Ca^{2+}-free and $Mg^{2+} \times 0{\cdot}5$ sea water for 33 min. (*c*) *Calliactis* column preparation ('low column') treated with Mg^{2+}-free and $Ca^{2+} \times 6$ sea water (i.e. Mg^{2+} replaced by Ca^{2+}) for 35 min. (*d*) *Calliactis* column preparation ('low column') treated with 0·54 NaCl only for 62 min. Time traces, 15 min.

The results with ions also show the limits of performance of the column musculature. Direct effects, as with Mg^{2+}-free sea water and excess K^+, seldom appear in less than 2 min. and then shortening is slow and usually stepwise (Figs. 1 *a*, 3 *b*), producing a state of high tonus reached over several minutes. In the most enhanced activity observed, contractions occur about once every 2 min. Responses to stimuli are less sensitive to the action of ions than might have been expected. Inhibitory treatments depress and delay responses, but their size and latency are not affected much by excitatory treatments like excess K^+ and reduced Mg^{2+}, though perhaps excess Ca^{2+} can double the size and speed of the response (Fig. 2*b*). It is clear that in the column the excitable and contractile system is basically slow and cannot be made to function much more quickly than it does under normal conditions.

Table 3. *Effects of multiple ion excess or deficiency on column preparations of* Calliactis

	Effects of treatments				
	Direct contractions	On tonus	On activity	On responses	Resemblances to effects of single ions
$K^+ \times 4$ $Ca^{2+} \times 4$	++	+++	+	++	Like excess K^+
$K^+ \times 4$ $Mg^{2+} \times 2$	+	o	−−	−−	Like excess Mg^{2+}
$Ca^{2+} \times 2$ $Mg^{2+} \times 1{\cdot}5$	o	o	−−	−−	Like excess Mg^{2+}
$Ca^{2+} \times 6$ $Mg^{2+} \times 2{\cdot}5$	o	−	→ Ab	→ Ab	Like excess Mg^{2+}
K^+-free Ca^{2+}-free	o	−	→ Ab	→ Ab	Like Ca^{2+}-free
K^+-free Mg^{2+}-free	++	++	++	Unobs.	Like Mg^{2+}-free
Ca^{2+}-free Mg^{2+}-free	+++	+++	+++	Unobs.	Like Mg^{2+}-free
$Ca^{2+} \times 0{\cdot}5$ $Mg^{2+} \times 0{\cdot}5$	+++	++	++	Unobs.	Like Mg^{2+}-free
Ca^{2+}-free $Mg^{2+} \times 0{\cdot}5$	o	−	→ Ab	→ Ab	Like Ca^{2+}-free
$Ca^{2+} \times 0{\cdot}5$ Mg^{2+}-free	+++	+++	+++	Unobs.	Like Mg^{2+}-free
$Ca^{2+} \times 6$ Mg^{2+}-free	+++	+++	+++	Unobs.	Like Mg^{2+}-free
NaCl only	+	+	→ Ab	−−	

(For explanation of + and − notation see text. No effect recorded as o. → Ab indicates depression of activity or responses leading to abolition. 'Unobs.' = unobservable.)

THE ACTION OF DRUGS

The drugs which were tested on the column preparations were mostly amines of general biological occurrence and widespread activity. They will be treated in four groups:

(1) acetylcholine and drugs acting at cholinergic junctions;
(2) sympathomimetic amines and associated substances;
(3) indolealkylamines and their associates;
(4) histamine, and some miscellaneous substances.

A standard pattern of test was employed in each case. The drug was introduced into the bath in concentrations of 1×10^{-5} and 1×10^{-4} as trial doses. Separate experiments were usually carried out to study the effect of a treatment on the inherent activity without stimulation and on the responses to electric shocks. If a substance were totally ineffective in four tests at these concentrations, no further tests were carried out. If, however, there were signs of even slight activity, further tests were conducted at these and other concentrations. Some substances giving negative results in the first tests were also given additional trials because of their importance and activity on nerve and muscle in other animals.

The results of the drug experiments are presented in Tables 4, 5 and 6 and they are expressed quantitatively in the same way as the ion effects above. A glance at these tables shows the following general points: (1) The great majority of drugs and substances employed in these tests had no detectable effects on the column preparations. (2) No drugs acted at dilutions comparable to the minimum effective doses in most other systems; the minimum effective dose in this case was 1×10^{-6} and most of the effects were observed at concentrations of 1×10^{-5} or $1 - 10^{-4}$. (3) The responses to stimuli were very little influenced by any of the drugs used in the tests. (4) No substances were found which blocked, or more than slightly diminished, the inherent activity of the preparations. A detailed description of the results with the different classes of drugs follows.

(1) *Acetylcholine and drugs acting at cholinergic junctions*

The experiments showed clearly that the movements and responses of the column preparations, like the quick responses of the whole animal (Ross, 1945), are unaffected by concentrations of acetylcholine (chloride—Roche) up to 1×10^{-4}. Introducing eserine (physostigmina or physostigmin sulphas—B.D.H.), at the same time or before acetylcholine, did not alter this negative result. Eserine itself, in very high concentrations (1×10^{-3}), caused a slow rise in tonus but did not alter the frequency or size of the slow movements and had no effect on the responses to stimulation. Atropine (sulphate—B.D.H.) and nicotine (acid phosphate—B.D.H.) at 1×10^{-4} were ineffective, as were the specific neuromuscular and ganglionic blocking agents, curare (*d*-tubocurarine chloride—Savory and Moore) and hexamethonium (tartrate—May and Baker). One other ester or choline was tested, carbachol (carbamyl choline chloride—B.D.H.) at 1×10^{-4} but this, like acetylcholine, had no effect. Tetramethylammonium (iodide—B.D.H.), known to be present in extracts of *Actinia equina* (Ackermann, Holtz & Reinwein, 1923) though not identified in extracts of *Calliactis* and *Metridium* (Mathias, Ross & Schachter, 1960), was also ineffective.

Thus, there is no suggestion that this class of substance is important in slow neuromuscular activity in *Calliactis* or *Metridium*. Clearly, the sea anemones, and perhaps the whole Phylum Coelenterata, do not employ acetylcholine as a neuromuscular transmitter or neurohumour (Bacq, 1947).

(2) *Sympathomimetic amines and associated substances*

Unlike the earlier tests with adrenaline on the quick response of whole *Calliactis* and *Metridium* (Ross, 1945), these experiments on the column preparations showed that adrenaline (chloride—Parke Davies; acid tartrate—B.D.H.) has an action on the column preparations. It causes direct contractions and a state of maintained tonus in which the musculature is shortened almost to its full extent. These effects

Table 4. *Effects of acetylcholine and associated substances on column preparations of* Calliactis *and* Metridium

		Effects of treatments			
Drug	Dose	Direct contractions	On tonus	On activity	On responses
Acetylcholine	5×10^{-5}	o	o	o	o
	1×10^{-4}	o	o	o	o
Ach (eserinized)	1×10^{-4}	o	+	o	+
	6×10^{-4}	o	+	+	o
Carbechol	1×10^{-4}	o	o	o	o
Choline	1×10^{-4}	o	o	o	o
Physostigmine	1×10^{-4}	o	o	o	o
	1×10^{-3}	o	+	o	o
Atropine	1×10^{-4}	o	+	o	o
D-tubocurarine	1×10^{-4}	o	o	o	o
Nicotine	1×10^{-4}	o	+	o	−
Tetramethyl-ammonium iodide	1×10^{-4}	o	o	o	o
Hexamethonium	1×10^{-4}	o	o	o	o

(For explanation of + notation see text. o indicates no effect)

appear in less extreme form at concentrations as low as 1×10^{-6} (Fig. 6*a*); at 1×10^{-4} they are striking (Fig. 6*c*). At higher concentrations, the small contractions in tonus follow each other more frequently than the full contractions in the preliminary record. At concentrations too low to cause direct effects (1×10^{-6} to 1×10^{-8}), the size of the response and its latent period did not differ appreciably from the controls.

Table 5 summarizes the results for this class of substances and shows that the adrenaline effect is unique. Noradrenaline (acid tartrate—Bayer) has no significant effect. Tyramine (hydrochloride—B.D.H.), previously shown to have profound effects on the size of the quick response (Ross, 1945) and now known to have similar effects on excised sphincters (Ross, 1960), is only slightly active on column preparations. Ephedrine (hydrochloride—B.D.H.), nor-sympatol, 1-*p*-sympatol, 3-hydroxytyramine (gifts of Dr H. Blaschko) and amphetamine (benzedrine), which might have been expected to display adrenaline-like effects, had no action on the tonus or activity of the preparations or their responses to stimulation. The only substances of this group which did affect the preparations in an adrenaline-like way were isoamylamine (B.D.H.), which was moderately active, and phenylethylamine (hydrochloride—Roche) which was slightly so (Fig. 6*d*).

Although cocaine (hydrochloride—B.D.H.) enhances the quick facilitated response of the whole animal (Ross, 1945), it has no effects on the tonus or activity of the column preparations or on the response to stimuli. Moreover, it does not enhance the response to adrenaline or make the preparations more sensitive to it. Thus the potentiating action of cocaine in relation to adrenaline and the sympathetic system in the vertebrates is completely lacking here.

The effects of several adrenergic blocking agents were investigated. They included ergotoxine (ethanesulphonate—Burroughs Wellcome). 933 F (piperidinomethylbenzodioxane), dibenamine (gift of Dr H. Blaschko) and regitin (methanesulphonate—CIBA). With the exception of 933 F, these substances are only slightly soluble in sea water and the doses could not be exactly determined; they were probably less than 1×10^{-5}. 933 F at 1×10^{-4} and saturated solutions of the others, however, had no depressant or inhibitory effects on the responses to stimulation, on the activity and tonus of the preparations or on the response of the preparation to adrenaline at 1×10^{-5}.

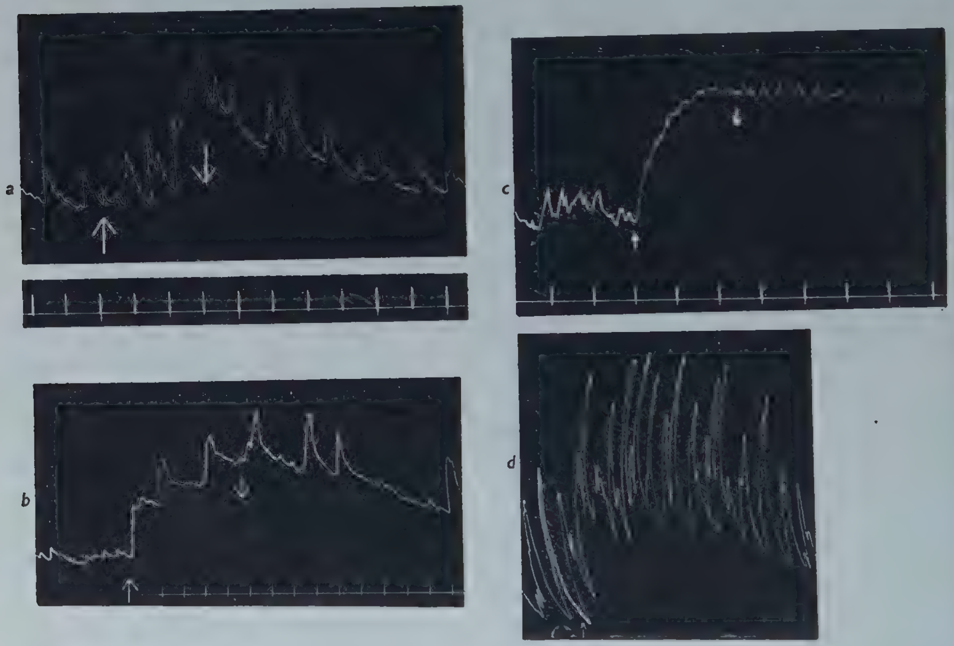

Fig. 6. (*a*) *Calliactis* column preparation ('mid-column') treated with adrenaline chloride (1×10^{-6}) for 45 min. Time trace, 15 min. (*b*) *Calliactis* column preparation ('mid-column') treated with adrenaline chloride (1×10^{-5}) for 30 min. Time trace, 6 min. (*c*) *Calliactis* column preparation ('mid-column') treated with adrenaline chloride (1×10^{-4}) for 30 min. Time trace, 15 min. (*d*) *Calliactis* column preparation ('mid-column') treated with phenylethylamine (1×10^{-4}) for 90 min. Time trace, 12 min.

(3) *Indolealkylamines and their antagonists*

There is little to add here to the earlier brief paper on the action of tryptamine and 5-hydroxytryptamine (5-HT) on the column preparations of *Calliactis* and *Metridium*. Tryptamine (hydrochloride—Roche and B.D.H.) at concentrations of 1×10^{-4} causes direct contractions and maintains a state of high tonus in the preparations without having much effect on activity (Fig. 7*a*). At lower concentrations the contractions are bigger and at higher concentrations, when the muscle is much shorter due to the direct effects, the latent period of the response to stimulation is reduced. 5 HT (serotonin creatinine sulphate—Roche), on the other hand, has little effect on these preparations.

Table 5. *Effects of sympathomimetic amines and associated substances on column preparations of* Calliactis *and* Metridium

		Effects of treatments			
Drug	Dose	Direct contractions	On tonus	On activity	On responses
Adrenaline Cl	1×10^{-7}	o	o	o	o
	1×10^{-6}	+	+	+	o
	1×10^{-5}	++	++	+	o
	1×10^{-4}	+++	+++	+	Unobs.
Adrenaline bitartrate	1×10^{-5}	+	+	o	o
	1×10^{-4}	+++	+++	+	Unobs.
Noradrenaline	1×10^{-5}	o	o	o	o
	1×10^{-4}	o	+	o	o
Dopamine	1×10^{-4}	o	o	o	o
L-*p*-Sympathol	1×10^{-4}	o	o	o	o
Norsympathol	1×10^{-4}	o	o	o	o
Tyramine	1×10^{-5}	o	o	o	o
	1×10^{-4}	o	+	o	o
	$2{\cdot}5\times10^{-4}$	+	+	o	o
3-Hydroxy-tyramine	1×10^{-4}	o	o	o	o
Phenylethylamine	1×10^{-4}	o	+	+	o
Ephedrine	1×10^{-4}	o	o	o	o
Amphetamine	1×10^{-4}	o	o	o	o
Isoamylamine	1×10^{-4}	+	+	o	o
Cocaine	1×10^{-4}	o	+	+	o*
Ergotoxine	$<1\times10^{-4}$	o	o	o	o†
Dibenamine	$<1\times10^{-4}$	o	o	o	o†
Hydergine	1×10^{-4}	o	+	o	−†
Yohimbine	$<1\times10^{-4}$	o	o	o	o†
933 F	1×10^{-4}	o	++	o	o†
Regitin	1×10^{-4}	o	o	o	o†

(For explanation of + notation see text. o indicates no effect. 'Unobs.' = unobservable due to effects on tonus and activity)

* No potentiation of response to adrenaline test.

† No effect on response to adrenaline test.

Other derivatives of tryptamine and substances with related effects have been tested on the column preparations (Table 6). 5-Methoxytryptamine (Roche) caused direct contractions and a rise in tonus and was almost as effective as tryptamine at the same concentration (Fig. 7*b*). 5-Benzyloxytryptamine (Roche), at an undetermined concentration (only slightly soluble in water) also caused a rise in tonus. It is clear that these preparations are generally sensitive to certain types of indolealkylamine molecules, but it would require a more extensive survey than could be made here to indicate what features the effective substances of this group have in common.

Substances which affect serotonin activity elsewhere have also been tested. Lysergic acid diethylamide (L.S.D.), which antagonizes the response to 5 HT in a number of muscle preparations, and reserpine which releases 5 HT from brain and other tissues (Page, 1958), had no detectable effects on these preparations. Thus, as with adrenaline, no clear parallel exists between the sensitivity of the anemone column musculature to tryptamine and some of its derivatives and the effects on

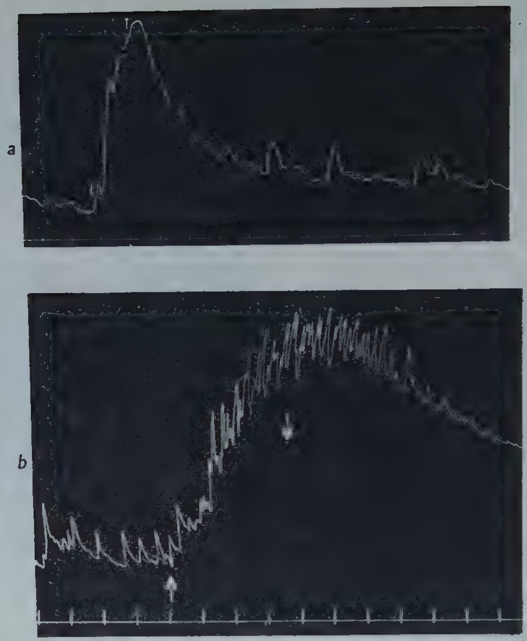

Fig. 7. (*a*) *Metridium* column preparation ('mid-column') treated with tryptamine hydrochloride (1×10^{-4}) for 4 min. Time trace, 3 min. (*b*) *Calliactis* column preparation ('low column') treated with 5-methoxytryptamine (1×10^{-4}) for 60 min. Time trace, 15 min.

nerve and muscle in other animals. Welsh's (1957) suggestion that 5-HT is a neurohumour in a number of invertebrates cannot be applied to anemones, although *Calliactis* contains large amounts in the tissues lining the coelenteron (Mathias, Ross & Schachter, 1957).

(4) *Histamine and other active or widely distributed substances*

The remaining substances whose effects are summarized in Table 6 are a miscellaneous group chosen for their effects on other preparations or because they are known to occur in a wide range of animals, especially amongst marine invertebrates. They include histamine (acid phosphate—Burroughs Wellcome), strychnine (sulphate—B.D.H.), adenosine triphosphate (Light), γ-amino-butyric acid (Roche), and guanidine (sulphate—B.D.H.). With the exception of ATP, which had moderate contractile and tonic effects, all these substances were totally ineffective at concentrations of 1×10^{-4}. The failure of these substances emphasizes once again that the actininian neuromuscular system has few pharmacological links with nerve and muscle in other animals.

DISCUSSION

Discussion of the results will be confined to the three topics: comparative aspects of the ion and drug effects; site of action of effective substances; the bearing of the results on the problem of rhythmical activity. The mechanism of slow responses

Table 6. *Effects of indolalkylamines and some other substances on column preparations of* Calliactis *and* Metridium

		Effects of treatments			
Drug	Dose	Direct contractions	On tonus	On activity	On responses
Tryptamine	1×10^{-6}	o	o	o	o
	1×10^{-5}	o	o	o	+
	5×10^{-5}	++	+	o	+
	1×10^{-4}	+++	+++	+	++
5-Hydroxy-tryptamine	1×10^{-5}	o	o	o	o
	1×10^{-4}	o	+	o	+
	$2\cdot5 \times 10^{-4}$	o	+	o	+
5-Methoxy-tryptamine	1×10^{-4}	+	+	+	+
5-Benzyloxy-tryptamine	$< 1 \times 10^{-4}$	o	+	+	o
Lysergic acid	1×10^{-4}	o	o	o	o*
Reserpine	$< 1 \times 10^{-4}$	o	o	o	o*
Histamine	1×10^{-4}	o	o	o	o
	$2\cdot5 \times 10^{-4}$	o	+	o	o
Cysteinamine	1×10^{-4}	o	o	o	o
Ethylamine	1×10^{-4}	o	o	o	o
Strychnine	1×10^{-4}	o	+	—	o
Adenosine triphosphate	1×10^{-4}	+	+	o	o
γ-Aminobutyric acid	1×10^{-4}	o	o	o	o
Guanidine	1×10^{-4}	o	o	o	o

(For explanation of + notation see text. o indicates no effect)
* No effect on response to tryptamine test.

to stimulation will be discussed together with slow responses of the sphincters in the next paper (Ross, 1960).

The effects of ions on contractile rhythms in different animals show few consistent patterns. Prosser (1950) has summarized the data from experiments on a good many vertebrates and invertebrates without finding any common features distinguishing, for instance, neurogenic from myogenic hearts, or marine from non-marine species. Comparing the anemone results with Prosser's table and with data on other organs, for example, *Cucumaria* cloaca (Wells, 1942), *Arenicola* extrovert (Wells & Ledingham, 1942), shows that some of the effects obtained on the column preparations are not unique. The lively action of Mg^{2+}-free sea water on tone and rhythm is found also in *Ostraea*, *Aplysia* and *Arenicola*. The effects of excess and lack of Ca^{2+} are more unusual. Only in the frog heart does Ca^{2+}-lack depress the rhythm as it does in the column preparations.

The only close parallel with the anemone results is in another coelenterate, the medusa *Cassiopea*, studied by Mayer (1906). He described the effects of various artificial sea waters on the rate of pulsation of *Cassiopea* before and after the removal of the marginal sense organs which initiate the rhythm in the normal medusa. The disk deprived of sense organs gave results that correspond very closely with the effects of similar sea waters on the column preparations. With the whole medusa there were differences in the effects of sea water lacking both Ca^{2+} and Mg^{2+} and sea water lacking both K^+ and Mg^{2+} (Table 7). Perhaps there is a suggestion here that column preparations correspond with the medusa without its marginal sense organs, i.e. without nervous pacemaker, and that the ion effects are due to actions on the contractile system itself.

Table 7. *Data (from A. G. Mayer) on the action of ions on pulsation of the medusa* Cassiopea *compared with effects on the activity of column preparations of* Calliactis

	Cassiopea		
	Whole medusa	Without sense-organs	Calliactis
NaCl only	+ + → −	− → Ab	− → Ab
K^+ and Mg^{2+}-free	o → −	+ → −	+ +
K^+ and Ca^{2+}-free	− → Ab	Ab	− → Ab
Ca^{2+} and Mg^{2+}-free	+ + → −	+ + → −	+ + +
Mg^{2+}-free	+ + +	+ + +	+ + +
Ca^{2+}-free	− → Ab	Ab	Ab
K^+-free	o → −	Ab	− → Ab

(For explanation of + and − notation see text. o indicates no effect. → Ab indicates depression of activity or responses leading to abolition)

Information on the site of action of effective ion and drug treatments was obtained by the technique described above (p. 733) for distinguishing effects on the contractile system from effects on more remote sensory and nervous elements. When preparations consisting of a loop of column attached to a longitudinal strip of column were treated differentially with excess K^+, Mg^{2+}-free sea water, adrenaline or tryptamine, effects were obtained only when these substances were brought into contact with the contractile loop. No effects occurred when the strip alone was treated. This shows that the effects arise either in the muscle itself or in that part of the nerve net in immediate contact with the muscle.

The drug effects show no obvious parallels with other animals. Although the preparations are sensitive to adrenaline and tryptamine, substances which influence the activity of these two drugs on other muscles have no corresponding effects on the column preparations. Therefore, the adrenaline effect cannot be assumed to have a functional significance linking it with the action of adrenaline on sympathetic effectors. Yet because the few substances that are effective tend to be sympathomimetic in their effects on vertebrates (cf. Ross, 1945) some affinity between the two is indicated. In this connexion, Östlund's (1954) survey of catechol amines in lower animals is important. In extracts of *Metridium* prepared by a specific catechol adsorption method he detected a substance on paper chromatograms of which the spray reactions and pharmacological properties were those of a catechol amine. It differed in R_f value from adrenaline, noradrenaline, and dopamine, so he called it 'catechol-4'.

It is not possible from the results with ions and drugs to throw much light on the problem of the origin and maintenance of the slow periodic activity seen in isolated preparations. Simple enhancing and inhibitory effects on the rhythm were not found. Increased frequency of rhythm, particularly in Mg^{2+}-free sea water and with adrenaline (1×10^{-4}), was always associated with higher tone, and cessation of activity with relaxation. These observations are consistent either with a myogenic or neurogenic origin of the rhythm. However, there were two situations, K^+-free and NaCl-free sea waters, where depression and eventual cessation of the rhythm occurred without the abolition of responses to electrical stimulation. Thus the nerve net was excitable and could conduct, the muscle could contract, and yet there was no periodic activity. This is more in keeping with an external nervous pacemaker than with a rhythm built into the contractile mechanism itself. Ewer's (1960) approach to this problem, based on carefully designed stimulation experiments on column preparations, has provided more reliable information on the factors controlling the rhythm. Any further work with ions and drugs must take into account his demonstration of the interplay of excitation and inhibition in the rhythmical activities and the responses of these animals.

SUMMARY

1. Isolated preparations of the columns of *Calliactis* and *Metridium* take up a basic extended length and show repeated contractions at intervals of a few minutes to half an hour. The preparations respond to stimuli with slow movements whose latencies range from about 20 sec. to 2 min.

2. Direct contractions and higher tonus of the preparations are caused by excess K^+ and by Mg^{2+}-free sea water; excess -Ca^{2+} raises tone slightly.

3. Activity is greatly enhanced by Mg^{2+}-free water, and moderately enhanced by excess K^+ and excess Ca^{2+}. It is abolished by excess Mg^{2+} and Ca^{2+}-free sea water, but not by sea water free of both Ca^{2+} and Mg^{2+}. In K^+-free sea water and in Na^+-free sea water activity declines slowly and responses to stimuli still occur for at least 1 hr.

4. Except for some enhancement by excess Ca^{2+} and the depression with excess Mg^{2+} and Ca^{2+}-free sea water, responses to stimuli are not much affected by ions.

5. Acetylcholine and associated drugs, and also histamine, 5-hydroxytryptamine, and many other substances, have no significant effects on the tone, activity, or responses of column preparations.

6. Adrenaline raises tonus at a concentration of 1×10^{-6}; at 1×10^{-5} and above; it causes direct contractions, enhanced activity and high tonus, but it has no effect on responses to stimuli. Noradrenaline, dopamine, tyramine, and other related compounds, as well as various adrenergic blocking agents, are totally inactive.

7. Tryptamine, at concentrations of 1×10^{-4}, causes direct contractions, high tonus and enhanced responses to stimulation. 5-Methoxytryptamine has similar effects.

8. To be effective, treatments must be applied directly to the contractile part of the preparation. Applying the active ions and drugs to attached strips of column has no effect.

9. Some comparative and functional aspects of the results are discussed.

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THE EFFECTS OF IONS AND DRUGS ON NEURO-MUSCULAR PREPARATIONS OF SEA ANEMONES

II. ON SPHINCTER PREPARATIONS OF *CALLIACTIS* AND *METRIDIUM*

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INTRODUCTION

Ever since Pantin (1935) showed facilitation to be the outstanding physiological feature of the quick closing response of the sea anemone *Calliactis parasitica* there has been much interest in this response. It is unique in that a single stimulus has no mechanical effect; responses occur only to the second and subsequent stimuli within a frequency range whose lower limit is about one stimulus per 3·0 sec. (17° C.) where the response finally vanishes. There is also a remarkably regular relationship between frequency of stimuli and the size of the individual steps in the 'staircase'. Yet the mechanism remains mysterious; in spite of some indirect evidence that facilitation is a chemical process at the junctions, direct proof of this is lacking. This indirect evidence comes from studying the effects of temperature on *Metridium* (Hall & Pantin, 1937), of ions on *Calliactis* (Ross & Pantin, 1940), and of drugs and extracts on *Calliactis* and *Metridium* (Ross, 1945*a*, *b*, 1952). The general conclusion was that neuromuscular transmission is a two-process system, in which the first process, facilitation, is both a prerequisite and a modifier of the second process, excitation of the muscle.

The work described in the present paper repeats and extends the earlier studies using not whole animals but preparations containing muscles that in life close up the anemone, the marginal sphincters of *Calliactis and Metridium*. Although some results merely confirm what was seen in the whole animals, a number of new effects have been observed. The new information chiefly relates to the slow movements of sphincter preparations (Batham & Pantin, 1954; Ross, 1957*a*) which are overshadowed in the whole animal by the quick response.

METHODS

The methods employed were the same as those described in the preceding paper (Ross, 1960). Most of the experiments have been conducted on ring preparations of the margins of *Calliactis* and *Metridium* in which the main muscular element is the marginal sphincter. Movements have been recorded on simple spring levers since these muscles, under constant load, tend to stretch indefinitely. These levers permit some shortening but they mainly record changes in tension.

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With the sphincter preparations it was possible to use an improved method to test whether substances were having general neurosensory effects or local neuromuscular effects. A twochambered bath was constructed with a diaphragm of tambour rubber separating an inner from an outer compartment (Fig. 1). Sphincters with a full-length strip of column attached were placed in the inner chamber and when the strip of column had been allowed to relax, it was drawn through a tiny slit in the rubber diaphragm. The rubber closed around the joint between the strip and the sphincter and, in most cases, the fit was tight enough to prevent mixing of the fluids in the two chambers. By maintaining a higher level in the inner chamber, it was possible to see whether any leakage occurred. Electrodes were hooked into the end of the strip so that the preparation could be stimulated, as from the base of the column. The sphincter loop was then attached to a lever so that its activity and responses could be recorded.

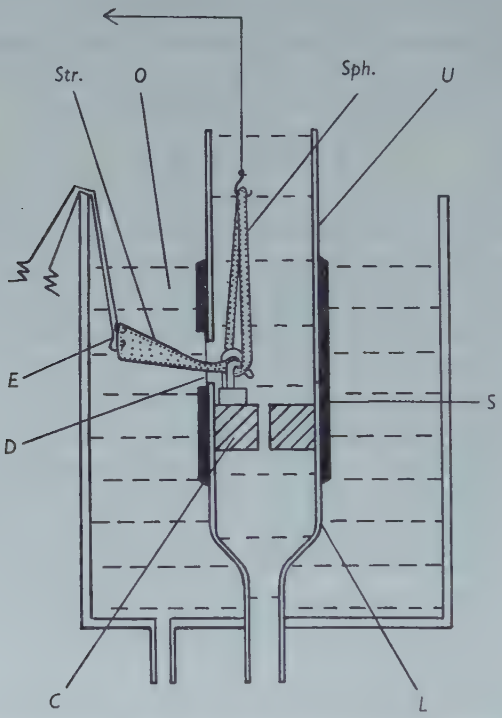

Fig. 1. Diagram of two-chambered bath for treating sphincter and attached strip of column independently. Consists of an inner bath formed by a notched upper tube (*U*) joined to a lower piece (*L*) by a thick rubber sleeve (*S*) with a central hole across which a thin rubber diaphragm (*D*) is fitted. The preparation is mounted on hooks set in the rubber cork (*C*) with the distal end of the sphincter loop (*Sph.*) attached to a recording lever. The strip of column (*Str.*) is drawn through a slit in the diaphragm into the outer bath (*O*) and, when stretched out by weights, stimulating electrodes (*E*) are inserted.

By this technique it was possible to treat the sphincter region alone by introducing substances into the inner bath and also to record its responses to indirect stimulation. Alternatively, by introducing substances into the outer chamber, the column alone could be treated. By comparing the results in the two situations it could be discovered whether treatments were exerting their effects by acting on or near the sphincter, or whether they were acting on sense organs and/or the conducting nerve net and only affecting the sphincter indirectly.

THE *CALLIACTIS* SPHINCTER PREPARATION

The properties of the *Calliactis* sphincter preparation have been fully described in an earlier paper (Ross, 1957*a*). In the absence of stimulation the preparation, like skeletal muscle, is quiescent. To electrical stimuli at higher frequencies these preparations give admirable quick faciliated contractions to every stimulus after the first. They also give slow responses, which may surmount the quick responses, but which are best seen after a number of stimuli at frequencies too low to cause quick contractions. Examples of these responses are seen in many of the subsequent figures.

The results are presented in a series of tables, each column indicating the effects of the treatments on one property of the sphincter preparation. As before, an attempt has been made to express these effects in a roughly quantitative way. Direct contractions can be easily identified as normally the preparation is quite inactive. The estimate of the number of direct contractions is based on an arbitrary rating in which '+' indicates up to 10 per hour, '++', from 11 to 20 per hour, and '+++' more than 20 per hour. Tonus is reckoned as '+' if tension is increased by up to 10% of full contraction, as '++' for an increase from 11 to 20% and '+++' for an increase in tension of more than 20%. If responses to single stimuli did occur, a 20% frequency of such responses was rated '+', 21–40% as '++' and more than 40% as '+++'. Changes in responses to stimulation were rated as '+' or '–' for a 20% increase or decrease in magnitude, '++' or '– –' for a 21–40% change and '+++' or '– – –' for changes greater than 40% in either direction.

The action of ions

The effects of various ion treatments are summarized in Table 1. There is no need to describe them fully, since in most respects they confirm the earlier results on whole animals (Ross & Pantin, 1940).

K^+ causes frequent direct contractions and some responses to single stimuli (Fig. 2*a*, *b*), but the most consistent effect is an early enhancement of the quick response to stimulation (Fig. 2*b*). There is no sign, however, that K^+ causes direct slow contractions or affects the size of the slow response. The tendency for K^+ to increase tonus seems to be due largely to its effect in prolonging contractions and inhibiting relaxation. Within a short time (15 min. with $K^+ \times 4$) all responses become depressed and eventually the preparation becomes inexcitable.

By contrast, Ca^{2+} has no direct contractile or tonic effects on the *Calliactis* sphincter preparation; however, it causes a huge enhancement of the response to stimulation but without changing the shape of the response (Fig. 3*a*). One effect of Ca^{2+} not observed with whole animals is seen in Fig. 3*b*, viz. a tendency to respond with a small movement to every stimulus, including the first of a series. These small movements make it difficult to see whether Ca^{2+} is having any effects on the slow response. The records in Fig. 3*c* show slow responses recorded on a very slow drum to twenty-five stimuli separated by intervals of 5·5 sec. at 22° C. Before treatment the latency was standard at about 150 sec., but with $Ca^{2+} \times 4$ after 24 min. the response began on the 5th stimulus, i.e. with a latency of about 25 sec. and the final contraction was vastly bigger. Close examination showed, however, that this effect was due to the summation of tiny contractions on each stimulus and was not the typical smooth slow contraction at all, although it developed into the usual type of slow contraction towards the end. There were a number of tests in which Ca^{2+} produced some shortening of the latency of the slow responses, but it is clear that Ca^{2+}, like K^+ exerts its main effect on the quick response and has only a slight effect on the slow response to stimulation.

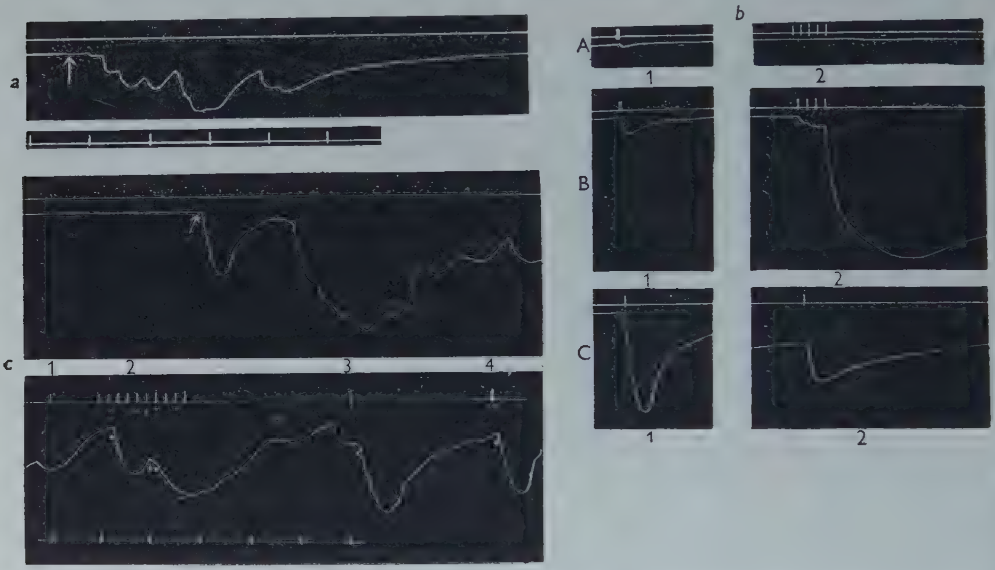

Fig. 2. (*a*) *Calliactis* sphincter preparation treated with $K^+ \times 4$. Continuous record without stimulation. Time trace, 5 min.

(*b*) Responses of *Calliactis* sphincter preparation. (A) In natural sea water before treatment: (1) to 2 stimuli at 1 in 1 sec. and (2) to 5 stimuli at 1 in 5 sec. (B) During treatment with $K^+ \times 2·5$: (1) to 2 stimuli at 1 in 1 sec. after 7 min. and (2) to 4 stimuli at 1 in 5 sec. after 21 min. Note the enhancement in (1) and quick responses to every stimulus with a huge response on stimulus 4 in (2). (C) To single stimuli after (1) 18 min. and (2) 29 min. treatment with $K^+ \times 2·5$.

(*c*) *Calliactis* sphincter preparation treated with K^+-free sea water for 9 min. Continuous record, lower trace following directly from upper trace. Time trace, 30 sec. Stimuli on lower trace as follows: (1) single shock—no response; (2) 10 stimuli at 1 in 6·5 sec., responses indicated by dots; (3) and (4) 2 stimuli at 1 in 1·1 sec., responses only on stimulus 2 and indicated by dots.

The direct effects of K^+-free sea water on the sphincter preparation are very much like those of excess K^+, but with many contractions, and with a maintenance of

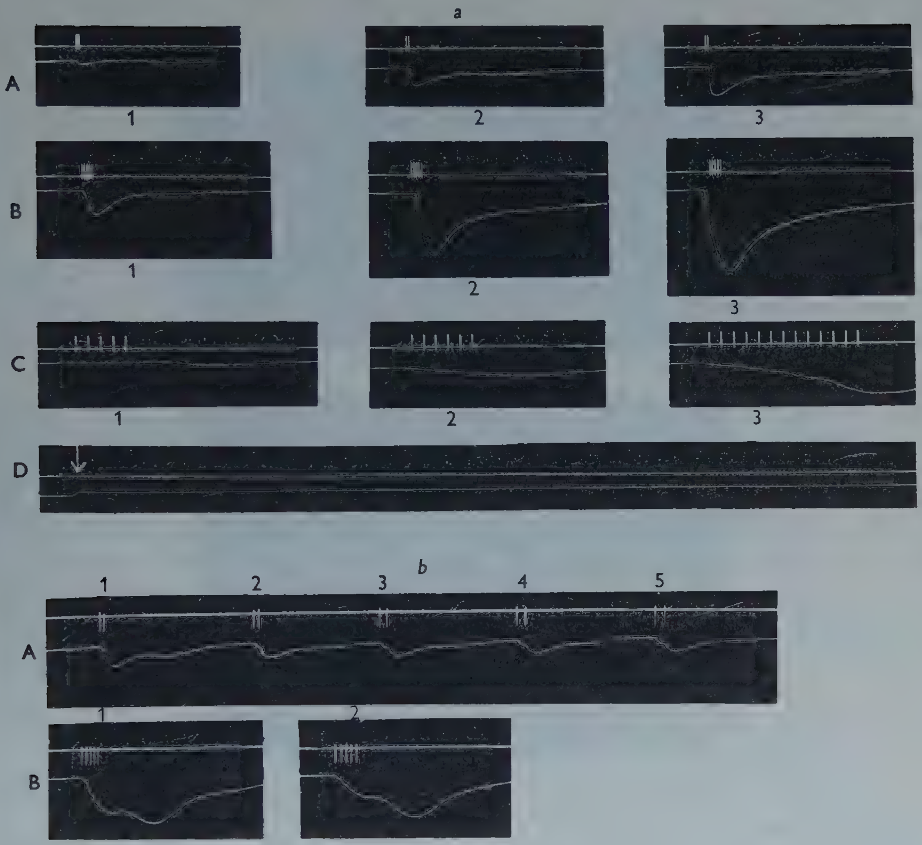

Fig. 3. (*a*) *Calliactis* sphincter preparation treated with $Ca^{2+} \times 4$. (A) Responses to 2 stimuli at 1 in 1 sec.: (1) before, (2) 11 min. and (3) 22 min. after introducing $CaCl_2$. (B) 'Staircase' responses to 5 stimuli at 1 in 1 sec.: (1) before, (2) 14 min. and (3) 25 min. after introducing $CaCl_2$. (C) Responses to stimuli at 1 in 5 sec.: (1) before (5 stimuli), (2) 31 min. (6 stimuli) and (3) 34 min. (13 stimuli) after introducing $CaCl_2$ (note small movements to every stimulus in (2) and (3)). (D) Continuous record for 6 min. after introducing $CaCl_2$ (note absence of direct effects).

(*b*) *Calliactis* sphincter preparation treated with $Ca^{2+} \times 4$ for approx. 2 hr. (A) Responses to 2 stimuli at intervals of: (1) 3·6, (2) 4·8, (3) 7·2 and (5) 8·4 sec. (B) Responses to 5 stimuli at intervals of: (1) 3·6 and (2) 4·8 sec. (Note responses to the first stimulus in A and B.)

tension for long periods yet without enhancing or depressing the responses to stimulation very much (Fig. 2*c*). Ca^{2+}-free sea water, however, is an active depressant; the muscle loses tone and within 5–10 min. ceases to respond at all. As with the column preparations, the whole effect is very similar to that of excess Mg^{2+}. The excitant effects with Mg^{2+}-free sea water on the column were reproduced on the sphincter. Uninterrupted quick contractions made it difficult to test responses to stimuli and to detect genuine responses to single stimuli (Fig. 3*d*).

Various combinations of excess and lack of two or more ions were also tested and showed the same relations here as with the column preparations, viz. that Mg^{2+} effects, both excess and lack, cannot be counteracted by increasing or decreasing

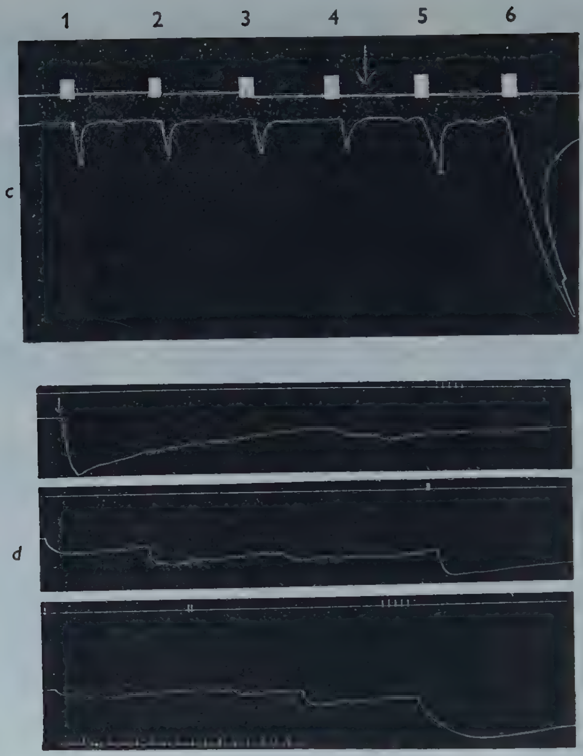

Fig. 3 (*c*) Responses of *Calliactis* sphincter preparation on a slow continuous record to 25 stimuli at 1 in 5·5 sec.; 15 min. elapses between each stimulation. Responses 1–4 preceded, and responses 5 and 6 followed 9 min. and 24 min., respectively, the introduction of $CaCl_2$ to make $Ca^{2+} \times 4$. Responses 1–4 were slow and had latencies of 145, 150, 150 and 150 sec. Responses 5 and 6 began with tiny twitches on the stimuli giving detectable responses of the lever at 80 and 25 sec., respectively, which developed into smooth contractions.

(*d*) *Calliactis* sphincter preparation treated with Mg^{2+}-free sea water for 24 min. Continuous record with responses to stimuli as indicated. Note tendency to maintain tension. Responses to stimuli are erratic, for example, final group of 5 stimuli at 1 in 5 sec. gave small twitch on stimulus 1, a big movement on stimulus 4 and no responses to stimuli 2, 3 and 5.

Ca^{2+} or K^+. Isotonic NaCl (lacking K^+, Ca^{2+} and Mg^{2+}), however, was a powerful excitant, producing big but infrequent quick contractions, and eventually a sustained full contraction.

All these treatments have been tested on the preparations with an attached strip of column in the two-chambered bath described above. The direct effects with excess K^+ and Mg^{2+}-free sea water, and the enhancement with excess K^+ and excess Ca^{2+}, were only obtained when the sphincter loop in the inner bath was exposed to the treatment. When the strip of column in the outer bath was treated, no direct effects, no enhancement and no responses to single stimuli were observed.

The action of drugs

Most of the drugs used on whole animals and on column preparations have been used again on *Calliactis* sphincter. A selection of the results is set out in Tables 2

Table 1. *Effects of excess and defect of cations on* Calliactis *sphincter preparations*

	Effects of treatments						
	Direct contractions				Responses to two or more stimuli		
	Quick	Slow	Tonus	Responses to single stimuli	Quick	Slow	Resemblances
Excess $K^+ \times 2$	o	o	o	o	+ +	?	.
$\times 4$	+	o	+	+	+ + +	+	.
$\times 8$	+ + +	o	+	+ +	+ + +	+	.
Excess $Ca^{2+} \times 2$	o	o	o	o	+	o	.
$\times 4$	o	o	o	+*	+ + +	o	.
$\times 8$	o	o	o	+ +*	+ + +	o	.
Excess $Mg^{2+} \times 2$	o	o	–	o	–	–	.
$\times 4$	o	o	– –	o	→ Ab	→ Ab	.
K^+-free	+ + +	+	+ + +	+ +	Unobs.	Unobs.	.
Ca^{2+}-free	o	o	–	o	→ Ab	→ Ab	.
Mg^{2+}-free	+ + +	o	+ +	o	+ +	– –	.
Na^+-free	o	o	o	+ + +	+ +	Unobs.	.
NaCl only	+ + +	o	+	o	+ +	o	
Mg^{2+}- and Ca^{2+}-free	+ + +	o	+ +	+	o	– –	As Mg-free
Mg^{2+}-free and 50 % Ca^{2+}	+ + +	o	+ +	+	o	– –	As Mg-free
Ca^{2+}-free and 50 % Mg^{2+}	o	o	–	o	→ Ab	→ Ab	As Ca-free

or explanations of + and – notation see text; o indicates no effect. → Ab indicates depression of activity or nses leading to abolition; 'unobs.' means unobservable due to effects of tonus and to direct contractions. * Responses ngle stimuli with excess Ca^{2+} are small.)

Table 2. *Effects of acetylcholine and associated substances and of sympathomimetic amines and various adrenolytic and sympatholytic drugs on* Calliactis *sphincter preparations*

		Effects of treatments					
		Direct contractions				Responses to two or more stimuli	
Drug	Dose	Quick	Slow	Tonus	Responses to single stimuli	Quick	Slow
Acetylcholine	1×10^{-4}	o	o	o	o	o	o
(eserinized)	6×10^{-4}	o	+	+	o	o	o
Eserine	6×10^{-4}	o	o	o	o	o	o
Nicotine	1×10^{-4}	o	o	o	o	o	o
Tetramethyl-ammonium iodide	1×10^{-4}	o	o	o	o	o	o
d-Tubocurarine	1×10^{-4}	o	o	o	o	o	o
Adrenaline	1×10^{-6}	o	+	o	o	o	o
	1×10^{-5}	o	+	+	o	o	+
	$2 \cdot 5 \times 10^{-5}$	o	+ +	+ +	o	o	+
	1×10^{-4}	o	+ + +	+ +	o	o	+ +
Noradrenaline	1×10^{-4}	o	+	o	o	o	o
Dopamine	1×10^{-4}	o	o	o	o	o	o
Tyramine	1×10^{-5}	o	o	o	o	+	o
	1×10^{-4}	o	o	o	+ +*	+ + +	o and –*
Phenylethylamine	1×10^{-4}	o	o	o	o	o	o
Isoamylamine	1×10^{-4}	+	+	o	o	+	+
Cocaine	1×10^{-4}	o	o	o	o	+	+
Ergotoxine	$< 1 \times 10^{-4}$	o	o	o	o	o	o
Dibenamine	$< 1 \times 10^{-4}$	o	o	o	o	o	o
993 F	1×10^{-4}	o	o	o	o	+	+

or explanation of + notation see text; o means no effect. * Responses to single stimuli with tyramine are small ffects on slow responses variable; see text.)

and 3. The experimental procedure was the same as that described for testing drugs on the column preparations.

Many results may be dismissed at once without comment since they are clear negatives. Thus acetylcholine and its associates, eserine, atropine, curare, etc., had no interesting effects. With whole animals these substances had no effects on facilitation and quick responses (Ross, 1945*a*), and we see now that they do not affect the slow responses either. Some other substances of widespread activity and occurrence, for example, histamine, γ-aminobutyric acid, tetramethylammonium iodide, are equally ineffective. In fact, only three substances had important effects: adrenaline, tyramine and tryptamine. These require a full description, although their main features may be seen from the tables.

Tyramine

Tyramine produces a remarkable enhancement of the responses of whole *Calliactis* (Ross, 1945*a*) and this appears also in the sphincter preparations (Fig. 4*a*). This enhancement, however, does not extend to the slow response. Indeed, the tyramine enhancement of the quick response is often accompanied by a depression of the slow response, where the latter appears in controls as a slow delayed movement surmounting an earlier quick contraction (Fig. 4*b*). It also depresses slow responses at lower frequencies, though at such frequencies small contractions to single stimuli generally appear (Fig. 4*c*). These experiments failed to confirm my earlier observation that tyramine causes big responses to single stimuli. Perhaps this was a mistake, or it may be that some factor outside the sphincter was involved. The tyramine effect is unique; 3-hydroxytyramine has no effects at all. In passing, it can be noted that the tyramine effect is very similar to the effect of excess Ca^{2+}, a point which will need to be discussed.

Tryptamine

A note has already been published (Ross, 1957*b*) on the action of tryptamine on both column and sphincter preparations of *Calliactis* and *Metridium*. Additional data now permit a more exact description of the effects of tryptamine on the activity and responses of the sphincter.

It has been confirmed that the outstanding feature of the tryptamine effect is a tendency for direct quick contractions to occur. Fig. 5*a* shows a section of a continuous record from 15 to 90 min. after introducing tryptamine (hydrochloride—Roche) at a concentration of 1×10^{-4}, in which contractions are occurring at the rate of about one per min. The appearance of the effect, both the size and the number of the contractions, is rather unpredictable but the effect is always of this same general type.

These direct effects of tryptamine coincide with a tendency to respond to single stimuli. Fig. 5*b* shows a record on a slow drum in which responses to single stimuli were obtained every time the preparation was tested.

The indirect effects of tryptamine on the size and latency of quick and slow contractions are not easy to determine. The tendency to respond to single stimuli makes it virtually impossible to test the effects of low-frequency stimulation and complicates the responses to stimuli at frequencies which give quick facilitated responses. Working with lower concentrations, at which direct effects are not so much in evidence (1×10^{-5}), it has been possible to see that tryptamine enhances the quick responses to stimuli. Occasionally with higher concentrations it is possible to get an impressive enhancement, but it never approaches the size of the tyramine enhancement at similar concentrations. The effects of tryptamine on the slow responses of the sphincter of *Calliactis* are less clear. Many further experiments have thrown doubt on the earlier published statement (Ross, 1957*b*) that tryptamine enhances the slow responses.

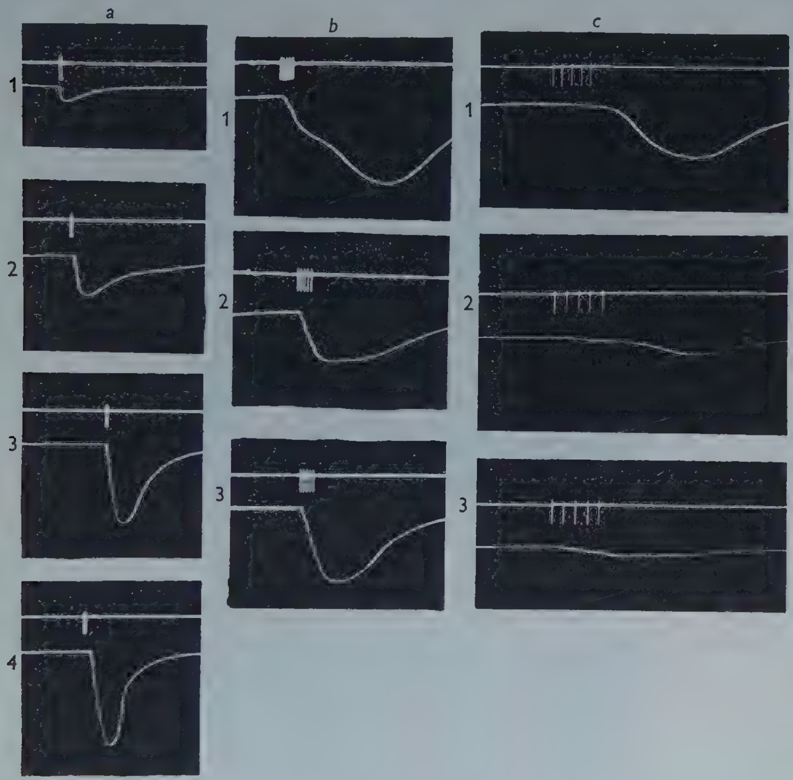

Fig. 4. *Calliactis* sphincter preparation treated with tyramine (1×10^{-4}).

(*a*) Responses to 2 stimuli at 1 in 1·2 sec.: (1) before, (2) 15 min., (3) 57 min. and (4) 110 min. after introducing tyramine.

(*b*) Responses to 5 stimuli at 1 in 2·4 sec.: (1) before, (2) 27 min. and (3) 73 min. after introducing tyramine.

(*c*) Responses to 5 stimuli at 1 in 6·1 sec.: (1) before, (2) 33 min. and (3) 88 min. after introducing tyramine.

Note great enhancement of facilitated responses in (*a*) and (*b*), disappearance of the slow responses in (*b*) and (*c*), and the appearance of small responses to every stimulus in (*c*).

Other indolealkylamines have been tested on the *Calliactis* sphincter preparation (Table 3). As reported earlier (Ross, 1957*b*), 5-hydroxytryptamine (5-HT) is inactive compared with tryptamine; direct quick contractions were seldom seen and only moderate enhancement of the quick response to stimuli occurs at concentrations of 1×10^{-4}. On the other hand, 5-methoxytryptamine (5-MT) caused direct quick contractions exactly like tryptamine, though the preparation seemed to be less sensitive to it than to the parent substance. The other derivative available, 5-benzyloxytryptamine (5-BT), like 5-HT, was ineffective. The drugs lysergic acid diethylamide and reserpine, inhibitor and releaser of 5-HT, respectively, on certain

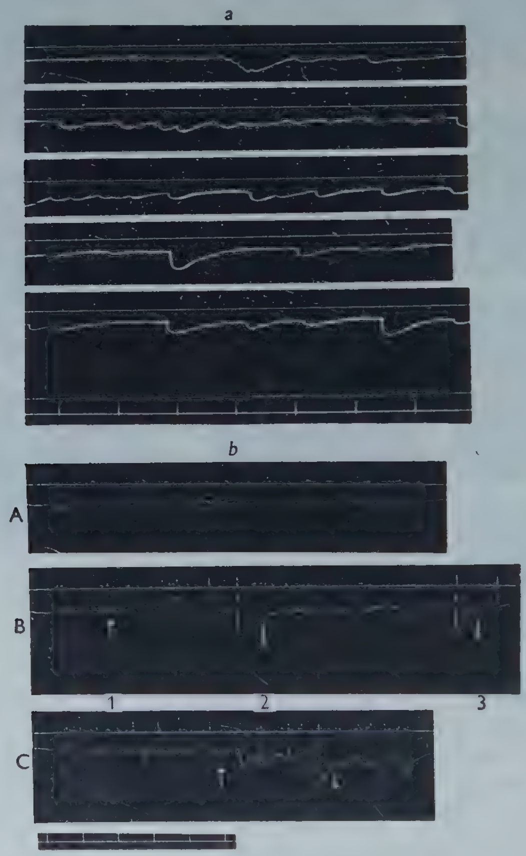

Fig. 5. (*a*) *Calliactis* sphincter preparation treated with tryptamine (1×10^{-4}) for 75 min. Continuous record without stimuli, beginning 15 min. and ending 90 min. after introducing the drug. Note the occurrence of quick contractions, about 1 per min., without the development of tension. Time trace, 2 min.

(*b*) Continuous record of *Calliactis* sphincter preparation on very slowly revolving drum. (A) In normal sea water for 1 hr. with single stimuli applied on four occasions indicated by signals. Note small slow movements and absence of responses to stimuli. (B) Continued from A with tryptamine (1×10^{-4}) for 25 min. and with single stimuli applied on three occasions during treatment (period of treatment indicated by arrows 1 and 2 and a second replacement of sea water by arrow 3) (note quick responses to stimuli during treatment). (C) Similar record to A and B with tryptamine hydrochloride (1×10^{-4}) for 20 min. and three single stimuli applied before, during and after treatment. White dots indicate quick movements on the stimuli. Note that there are no responses before the treatment, medium to large responses during treatment, and some small responses immediately after washing out the drug. Time trace, 6 min.

Table 3. *Effects of indolealkylamines and some other substances on* Calliactis *sphincter preparations*

Drug	Dose	Effects of treatments					
		Direct contractions				Responses to two or more stimuli	
		Quick	Slow	Tonus	Responses to single stimuli	Quick	Slow
Tryptamine	1×10^{-6}	o	o	o	o	o	o
	1×10^{-5}	+	+	o	o	+	+
	1×10^{-4}	+++	+	o	++	+++	+
5-Hydroxy-tryptamine	1×10^{-5}	o	o	o	o	o	o
	1×10^{-4}	o	o	o	o	+	+
5-Methoxy-tryptamine	1×10^{-4}	+	o	o	o	+	+
5-Benzyloxy-tryptamine	$<1 \times 10^{-4}$	o	o	o	o	o	o
Lysergic acid	—	o	o	o	o	o	o
Histamine	1×10^{-4}	o	o	o	o	o	o
Cysteinamine	1×10^{-4}	o	o	o	o	o	o
Ethylamine	1×10^{-4}	+	+	+	o	o	o
Strychnine	1×10^{-4}	o	o	o	o	o	+
γ-aminobutyric acid	1×10^{-4}	o	o	o	o	o	o
Guanidine	1×10^{-4}	o	o	o	o	o	o

(For explanations of + notation see text; o indicates no effect.)

other systems (Page, 1958), had no action on the sphincter preparation. Thus the pattern already seen with these indolealkylamines in the column preparations is repeated here, in which tryptamine stands as strongly excitatory, 5-M as excitatory, and 5-HT and 5-BT and drugs associated with 5-HT elsewhere, are all without effect.

Adrenaline

In whole animals adrenaline had no effect on responses to stimulation (Ross, 1945*a*), but we now know that it has a powerful action on the column musculature of *Calliactis*, causing direct contractions and the maintenance of high tonus (Ross, 1960).

Experiments on sphincter preparations have confirmed that adrenaline has no effect on the facilitated response and does not evoke direct quick contractions. However, it does cause slow contractions almost immediately after being introduced, at concentrations of 1×10^{-5} to 1×10^{-4}. These contractions look like the slow contractions of the sphincter to low-frequency stimuli and two examples are seen in Fig. 6. In Fig. 6*a* there is a single small quick contraction at about 3 min. In Fig. 6*b*, with a stronger dose, tension develops more quickly and irregularly. These irregularities are not due to quick contractions but to slight variations in the rate of slow contraction. Visual observation shows that a preparation giving quick responses, say when treated with excess K^+ or tryptamine, twitches visibly with every contraction. During the experiment with adrenaline seen in Fig. 6*b*, the preparation shortened steadily and smoothly without any twitching whatsoever.

Adrenaline also has some effect on slow responses to stimuli. The number of stimuli required to elicit a slow contraction at a definite frequency is usually considerably reduced by adrenaline. Fig. 6*a* shows a virtually unchanged quick response and an enhanced slow response with adrenaline at 1×10^{-5} and Fig. 6*b* shows an enhanced slow response immediately after adrenaline at 1×10^{-4}.

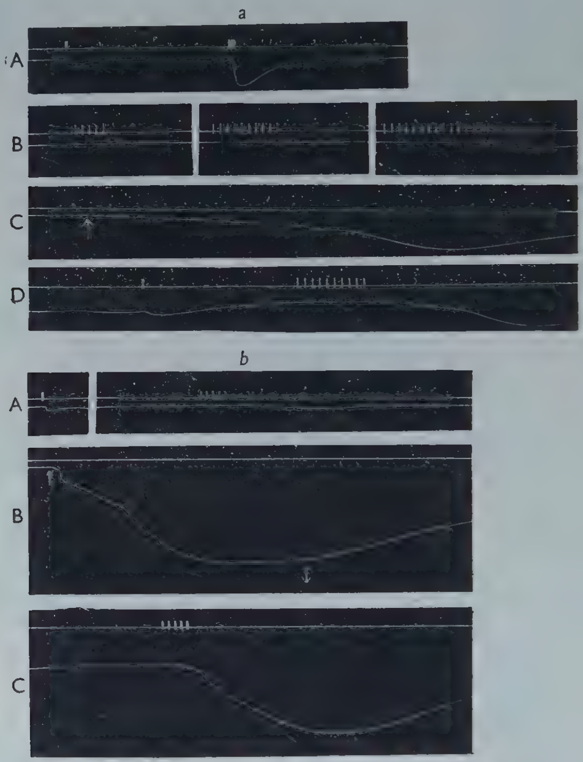

Fig. 6. (*a*) *Calliactis* sphincter preparation treated with adrenaline chloride (1×10^{-5}). (A) Responses to 2 and 5 stimuli at 1 in 1 sec. before treatment. (B) Responses to 5, 10 and 15 stimuli at 1 in 5 sec. before treatment (slow response only to 15 stimuli, no response to 5 or 10 stimuli). (C) Continuous record (duration 3 min.) during treatment with adrenaline (beginning at arrow) showing slow development of tension. (D) Continued from C with 2 stimuli at 1 in 1 sec. and 10 at 1 in 5 sec. (compared with controls, response to 2 at 1 in 1 sec. unchanged, but 10 at 1 in 5 sec., ineffective before treatment, gives a small slow contraction).

(*b*) *Calliactis* sphincter preparation treated with adrenaline chloride (5×10^{-5}). (A) Response to 2 stimuli at 1 in 1 sec. and to 5 stimuli at 1 in 5 sec. before treatment. (B) Continuous record during and after treatment (3 min. duration) with adrenaline (note slow and uneven development of tension). (C) Continued from above after washing out adrenaline with response to 5 stimuli at 1 in 5 sec., 6 min. after end of treatment (note great enhancement of slow response).

Apparently adrenaline has a selective action on the slow response mechanism of the sphincter. No other treatment with ions or drugs has had an effect of this kind. Fig. 7 shows the contrast, on a very slow drum, between the direct actions of tryptamine and adrenaline on the same preparation. This contrast is more marked if one is able to observe the preparation during the recording. Tryptamine contractions are visible twitches with a sharp beginning, and are followed by rapid relaxation without any maintenance of tension. Adrenaline contractions are gentle movements, barely visible, beginning almost imperceptibly on the record. Relaxation is slow and incomplete, and tension rises steadily. Quick movements may occur (Fig. 7 shows one example) but they only briefly interrupt the typical picture.

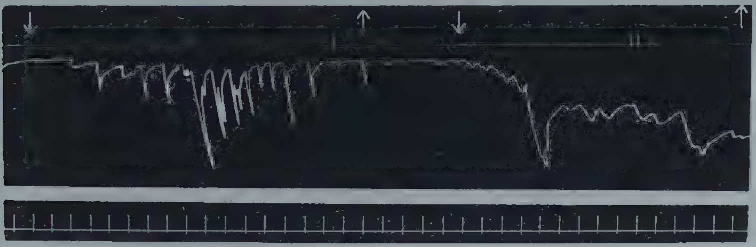

Fig. 7. Continuous record of *Calliactis* sphincter preparation treated with tryptamine hydrochloride (1×10^{-4}) for 65 min. (between arrows 1 and 2) and followed after a pause of 20 min. by adrenaline chloride (2×10^{-5}). Note quick twitches without maintenance of tension with tryptamine, and slow contractions and gradual development of tension with adrenaline. See text for full description. Signals indicate tests for responsiveness to single stimuli and white dots show corresponding positions on record (responses only with stimuli 2 and 3 on tryptamine record.) Time trace, 4 min.

Other sympathomimetic substances have been tested on the sphincter preparations, together with some adrenergic blocking agents, and cocaine, which potentiates many adrenaline effects in vertebrates. The results are shown in Table 2. None of these substances, not even noradrenaline and dopamine, have effects like adrenaline and most of them have no effects at all. Exceptions are: isoamylamine, which has moderately excitatory and enhancing effects; cocaine, which causes some enhancement of both quick and slow contractions; 933 F, which causes some enhancement of both kinds of response. The last substance was tested on whole *Calliactis* (Ross, 1945*a*) and gave greatly enhanced quick responses and some responses to single stimuli. In the isolated preparations the enhancement was less and no responses to single stimuli were seen.

The adrenaline inhibitors, the adrenergic blocking agents and the potentiating drug cocaine have no effect on the responses, or on the sensitivity of the preparations, to adrenaline. Thus, sensitivity to adrenaline is found without the allied pharmacological properties usually associated with adrenaline-sensitive systems. It is also worth noting that the effects of adrenaline and tyramine have little in common in anemones, whereas in vertebrates, although they differ in some respects, the two drugs have many identical effects.

The three most effective substances, tyramine, tryptamine and adrenaline, were all tested on the 'strip' preparation in the two-chambered bath. No effects were obtained when the drug was introduced into the outer chamber containing the strip of column (Fig. 1). Only when they were applied in the inner bath, directly in contact with the sphincter, did the characteristic effects appear. This rules out the possibility of general sensory or nervous excitation and indicates that these substances act on the sphincter itself, or on nervous elements in its immediate neighbourhood.

THE *METRIDIUM* SPHINCTER PREPARATION

Batham & Pantin (1954) studied the isolated sphincter of *Metridium* and found that it gives both quick and slow responses to stimuli. The quick response, however, differs from that of *Calliactis* sphincter. In *Calliactis* the quick response, if it is to occur at all, begins on the second stimulus of a series. In *Metridium* a quick response may occur only after several preliminary stimuli. In my experience four or five stimuli are usually required and it is rare to get a quick response from as few as two stimuli. Moreover, the quick response of *Metridium* sphincter is usually not a visible twitch as it is in *Calliactis*. To a series of stimuli, tension develops smoothly rather than in a series of discrete steps. In some ways it is better described as an early than as a quick response; the movement, although faster than the slow contraction, is much slower than the response of *Calliactis* sphincter and may continue to develop tension long after stimuli cease. Yet in spite of these differences, the frequency requirements of the quick facilitated response in *Metridium* sphincter are the same as in *Calliactis*, i.e. stimuli must not be more than 3 sec. apart or the quick response fails to appear at all.

Slow responses of *Metridium* sphincter preparations occur either as smooth sigmoid movements following the quick contractions after a definite latent period (usually about ½ min.), or as similar movements at longer latency to stimuli more than 3 sec. apart. This is like *Calliactis* sphincter. *Metridium* sphincter preparations, however, show a good deal of inherent slow activity which is not seen in *Calliactis*. This preparation, therefore, combines a number of features of the general column musculature with a partial development of a capacity for quick contraction; it may be said to occupy an intermediate position. For this reason it seemed desirable to examine the effects upon it of some of the treatments found to be effective on the other preparations.

Before undertaking these tests it was necessary to collect more information about the responses of the *Metridium* sphincter preparation. Batham & Pantin (1954) gave some data on this and I have compiled more from my experiments. These are given in Table 4.

One point of interest in this table is that, within the frequency range which elicits quick responses, good slow contractions can be produced in this preparation by small numbers of stimuli that elicit no quick responses, or very minute ones. Thus with five stimuli separated by intervals of either 1·1 or 2·2 sec., the quick response is only about one-quarter to one-eighth the size of the slow movement coming in about ½ min. later. With two or three stimuli at these frequencies large slow contractions are obtained although the quick contractions are insignificant. But with ten stimuli separated by intervals of 1·1 sec. almost the whole response is of the quick type (Table 4*b*). It seems clear from this that the two movements are alternative ways of developing tension in the same muscle and not separate muscle systems whose maximal contractions are additive. It is beyond the scope of this paper to discuss these figures but they show the quick and slow contractions sharing the task of responding to stimuli rather differently as compared with *Calliactis* sphincter. It would seem that the physiology of quick and slow contraction in these animals might be clarified by further studies on the *Metridium* sphincter preparation.

Table 4*a*. *Data on size (mm. on smoked record) and latency of quick and slow responses of two* Metridium *sphincter preparations to stimuli separated by intervals of* 1·1, 2·2 *and* 6·5 *sec.*

		Sphincter 1 (11–13°)				Sphincter 4 (12°)			
		Size		Latency		Size		Latency	
No. of stimuli	Frequency	Quick (mm.)	Slow (mm.)*	Quick (sec.)	Slow (sec.)	Quick (mm.)	Slow (mm.)*	Quick (sec.)	Slow (sec.)
5	1·1	2	12 (10)	6	59	3	6 (3)	6	43
10	1·1	9	13 (4)	6	62	7	11 (4)	7	42
5	2·2	1	8 (7)	11	80	<1	6 (5)	10	60
10	2·2	5	12 (7)	13	66	4	10 (6)	13	43
10	6·5	—	6	—	125	—	5	—	118

* Figures on size of slow responses are totals (quick + slow); net slow contraction in brackets (spring lever).

Table 4*b*. *Data on size (mm. on smoked drum) and latency of quick and slow responses to stimuli separated by intervals of* 1·1 *and* 2·2 *sec. in a* Metridium *sphincter preparation*

	Intervals between stimuli							
	1·1 sec.				2·2 sec.			
	Size		Latency		Size		Latency	
No. of stimuli	Quick (mm.)	Slow (mm.)*	Quick (sec.)	Slow (sec.)	Quick (mm.)	Slow (mm.)*	Quick (sec.)	Slow (sec.)
2	<1	10 (10)	4	55	0	5 (5)	—	90
3	4	13 (9)	4	40	<1	13 (13)	9	50
4	4	26 (22)	4	40	1	26 (25)	8	40
5	9	30 (21)	4	40	3	24 (21)	8	55
6	21	35 (14)	4	45	6	19 (13)	8	40
8	20	32 (12)	4	45	8	22 (14)	8	45
10	24	28 (4)	4	45	10	24 (14)	8	50

* Figures on size of slow responses are totals (quick + slow); net slow contraction in brackets (Gimbal lever).

The results of treating *Metridium* sphincter preparations with a selection of ions and drugs are shown in Table 5. These reinforce the general picture of ion and drug effects on column preparations and on *Calliactis* sphincter. Excess K^+ has excitant and tonic effects, but after at first enhancing quick responses it induces a general depression. Excess Ca^{2+} lacks direct effects but produces a spectacular and long-lasting enhancement of the quick response. Adrenaline causes direct contractions and increases tonus without affecting responses to stimuli, though at higher concentrations the direct effects make it difficult to obtain any exact records of the size or latency of responses. Noradrenaline showed signs of some direct action on *Metridium* sphincter, in contrast to its ineffectiveness on the other preparations, but it would require more work to decide how much importance should be attached to this observation. Tyramine showed no direct effects but again enhanced the quick response without any comparable effects on the slow response, though a slight effect on the latter was detected. Tryptamine, on the other hand, was followed by direct contractions and an increase in tonus; it also caused some enhancement of the response and shortened the latent period. 5-HT was ineffective.

Table 5. *Effects of excess* K^+ *and* Ca^{2+}, *and of adrenaline, noradrenaline, tyramine, tryptamine and 5-HT on* Metridium *sphincter preparations*

		Effects of treatment			
				Responses to stimuli	
Treatment	Dose	Direct contraction	Tonus	Quick	Slow
Excess K^+	×2	+	+	+	o
	×4	++	+++	++ then – –	o
Excess Ca^{2+}	×2	o	o	+	o
	×4	o	o	+++	o
Adrenaline	1×10^{-6}	o	+	o	o
	1×10^{-5}	++	++	o	o
	1×10^{-4}	+++	+++	Unobs.	Unobs.
Noradrenaline	1×10^{-4}	+	+	o	o
Tyramine	1×10^{-4}	o	o	+++	+
Tryptamine	5×10^{-6}	o	o	o	o
	1×10^{-5}	+	+	+	o
	1×10^{-4}	++	++	++	o
5-Hydroxy-tryptamine	1×10^{-5}	o	o	o	o
	1×10^{-4}	o	o	o	o

(For explanation of + notation see text; o indicates no effect.)

The effects of Ca^{2+}, tyramine and tryptamine in enhancing the responses are all similar in type. The quick response to a given number of stimuli is not only bigger in the treated preparation but it also begins earlier and is evoked by fewer stimuli. This 'facilitation number' (number of stimuli required just to elicit a contraction) seldom fell below three. Unlike Batham & Pantin (1954), I have never seen responses to single stimuli in untreated preparations, nor with any of the treatments used in these experiments.

Examples of these effects on *Metridium* sphincter preparations are seen in Figs. 8 and 9. It is interesting that although the timing of quick and slow contractions in the preparation is rather different from that in *Calliactis* sphincter, the effects of ions and drugs are roughly the same. The much slower and delayed 'quick' or 'early' contraction of *Metridium* sphincter apparently uses the same basic mechanism.

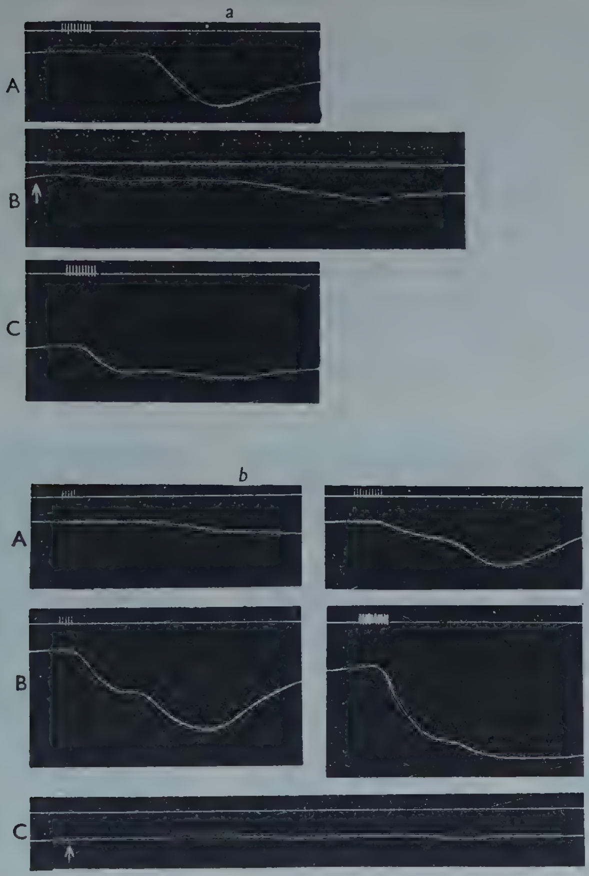

Fig. 8. (*a*) *Metridium* sphincter preparation treated with $\mathrm{K}^{+}\times 4$. (A) Response to 10 stimuli at 1 in 2·2 sec. before treatment. Note tiny 'early' (latency 17 sec.) and large later response (latency 55 sec.). (B) Continuous record for 25 min. after introducing KCl. (C) Response to 10 stimuli at 1 in 2·2 sec. during treatment at 12 min. Note high tonus, large 'early' (latency 5 sec.) and small 'late' (latency 60 sec.) responses.

(*b*) *Metridium* sphincter preparation treated with $\mathrm{Ca}^{2+}\times 4$. (A) responses to 5 and 10 stimuli at 1 in 2·2 sec. before treatment. Note tiny 'early' response and late response with 5 stimuli and larger 'early' and late responses with 10 stimuli (latencies 15 and 55 sec.). (B) Responses to 5 and 10 stimuli at 1 in 2·2 sec., 10 min. and 27 min. after introducing CaCl_2. Note large 'early' response (latency 7 sec.) with 5 stimuli followed by enhanced 'late' response (latency 55 sec.); and with 10 stimuli the 'early' response producing almost total contraction (latency 8 sec.) with only a small additional late movement (latency 60 sec.). (C) Continuous record for 2·5 min. after introducing CaCl_2 (note absence of direct effects).

DISCUSSION

When the effects of ions and drugs on both column and sphincter preparations are reviewed a certain pattern appears. Table 6 summarizes the data on several treatments which have marked positive effects. It shows: (1) the effects of K^{+} and tryptamine coincide closely; (2) the effects of Ca^{2+} and tyramine are almost identical; (3) the adrenaline effect is unique and without parallel amongst the ions. Some suggestions can be made about the nature of these effects.

In cases (1) and (2) above, it is reasonable to suppose that effects which are so similar must arise from the same basic actions on the system. Excess K^+ generally has depolarizing effects on excitable tissues. Therefore, its effects, and those of tryptamine, on anemone preparations are possibly due to an unspecific depolarizing and destabilizing action on the muscle membranes. Excess Ca^{2+} has no clear-cut general action on nerve and muscle. In anemone preparations excess Ca^{2+} and tyramine seem to enhance the contractility of the sphincter muscle when given the stimuli appropriate for quick contractions. Adrenaline evokes slow contractions of both types of preparation, and thus fulfils one of the requirements of a specific slow-excitor substance. The almost complete failure of other substances which act at adrenergic endings rules out any immediate suggestion that neuromuscular trans-

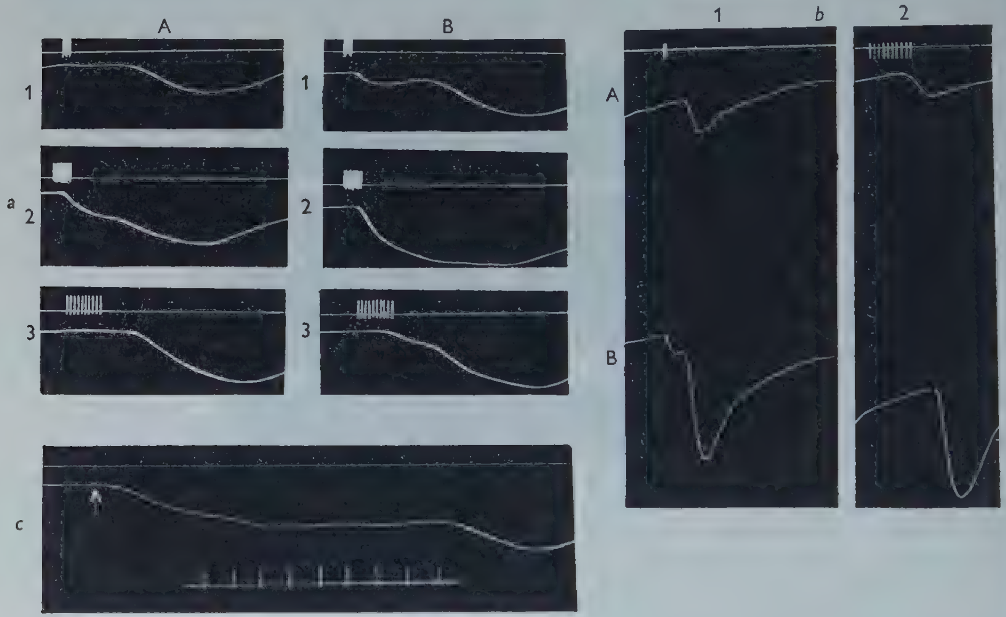

Fig. 9. (*a*) *Metridium* sphincter preparation treated with tyramine hydrochloride (1×10^{-4}). (A) Responses before treatment to: (1) 5 stimuli at 1 in 1·1 sec., (2) 10 stimuli at 1 in 1·1 sec. and (3) 10 stimuli at 1 in 2·2 sec. Note small and large 'early' responses in (1) and (2) respectively (latency 7 sec.) and later response in (1), (2) and (3) (latency 40 sec.). (B) Responses during treatment with tyramine to stimuli as in (1), (2) and (3) in A after 56, 81 and 68 min., respectively. Note larger 'early' responses in (1) and (2) (latency 5 sec.) and appearance of 'early' response in (3) (latency 17 sec.) and note 'late' response virtually unchanged in (1) and (3) but obliterated by maximal early response in (2) (latency 45 sec.) (spring lever recording).

(*b*) *Metridium* sphincter preparation treated with tryptamine hydrochloride (1×10^{-4}). (A) Responses before treatment: (1) to 5 stimuli at 1 in 1·2 sec. and (2) to 10 stimuli at 6·1 sec. Note single 'late' response in (1) (latency 65 sec.) and (2) (latency 55 sec.). (B) Responses during treatment with tryptamine to stimuli as in (1) and (2) in A after 48 and 70 min., respectively. Note double and enhanced response on (1) with latencies of 4 and 30 sec. for 'early' and 'late' responses, and enhanced response in (2) with latency of 50 sec. Isotonic lever recording shortening in a downward direction. The difference in level between the two traces represents the rise in tonus caused by the treatment.

(*c*) *Metridium* sphincter preparation treated with adrenaline chloride (1×10^{-4}) for 4 min. beginning with arrow. Note 'early' response to adrenaline (within 15 sec.). Time trace, 15 sec.

Table 6. *Summary of effects of most active ions and drugs on column and sphincter preparations of* Calliactis *and* Metridium *extracted from general tables*

		Column preparation (slow)				
		Direct effects			Responses	
Treatment	Concentration	Contractions	Tonus	Activity	Size	Latency
K^+ excess	×4	++	++	o	+	o
K^+ excess	×8	+++	+++	+	+	–
Tryptamine	1×10^{-4}	+++	+++	+	++	–
Ca^{2+} excess	×4	o	o	o	o	o
Ca^{2+} excess	×8	o	+	+	+	–
Tyramine	1×10^{-4}	o	+	o	o	o
Adrenaline	1×10^{-5}	++	++	+	o	o
Adrenaline	1×10^{-4}	+++	+++	+	Unobs.	Unobs.

		Sphincter preparation (quick and slow)						
		Direct effects			Quick response			
							Slow response	
Treatment	Concentration	Quick contractions	Slow contractions	Tonus	Responses to single stimuli	Size facilitated response	Size	Latency
K^+ excess	×4	+	o	+	+	+++	+	o
K^+ excess	×8	+++	o	+	++(L)	+++	+	–
Tryptamine	1×10^{-4}	+++	+	o	++(L)	+++	+	–
Ca^{2+} excess	×4	o	o	o	+(S)	+++	o	o
Ca^{2+} excess	×8	o	o	o	++(S)	+++	o	o
Tyramine	1×10^{-4}	o	o	o	++(S)	+++	o and –*	o
Adrenaline	1×10^{-5}	o	+	+	o	o	+	–
Adrenaline	1×10^{-4}	o	+++	++	o	o	++	–

(For explanation of + and – notation see text; o indicates no effect; L and S are large and small responses to single stimuli. * See text for explanation.)

mission in the slow response is an adrenergic mechanism. Yet Östlund's (1954) unidentified 'catechol-4' in *Metridium* may be linked functionally in some way with the adrenaline-sensitive slow contraction mechanism, and his finding should be followed up.

One of the main purposes of the work was to find out in what ways the quick and slow contractile systems resembled each other and in what other ways they differed. Table 6 shows that, allowing for the difference in time scale, they resemble each other in their responses to one treatment which directly excites nerve and muscle by a general depolarizing action, namely, excess K^+ (and probably tryptamine also since its effects are so similar). The two contractile systems are affected similarly by treatments which depress and abolish excitability, excess Mg^{2+} and Ca^{2+}-lack, though the latter may be due to a relative excess of Mg^{2+} when Ca^{2+} is withdrawn Ross (1960). Thus it seems likely that the quick and slow contractions are based on essentially similar conditions of membrane excitability, since their excitability is heightened and depressed by the same treatments in both preparations.

The two types of contraction differ profoundly in the effects on them of the treatments Ca^{2+} and tyramine, which affect the contractility of the muscles involved in quick responses to stimuli. Thus it seems that the main difference between the quick and slow contractions resides in the contractile mechanism, or in the mechanism by which contractions are evoked. The selective action of adrenaline in evoking slow contractions of both sphincter and column preparations reinforces the view that essentially different processes of initiating muscular contraction are involved in the two types of movement.

It is worth noting that the results provide no evidence that facilitation and slow contraction are affected in the same ways by the same treatments. The facilitation mechanism, if it is to be regarded as controlling the size of the quick response, seems to be most influenced by Ca^{2+} and tyramine, which affect slow contractions slightly if at all. Adrenaline, the only important slow-excitor substance found in this survey, is singularly without effect on facilitation. Therefore, if facilitation is carried out by a 'facilitator' and slow contraction is initiated by a 'slow-transmitter', these two substances and their modes of action must be quite distinct. Some preliminary work with extracts of *Calliactis* on both sphincter and column preparations has given evidence of potent substances with direct tonic effects on the slow contractile system. So far no effects of extracts on facilitation, or on slow responses to stimuli, have been detected. Work is in progress to confirm these preliminary results, and if they are confirmed, to find out what kinds of substances in the extracts are responsible for the effects observed in the preparations.

Although this work has shown that certain ions and drugs act selectively on the mechanisms of quick and slow contraction, these mechanisms seem to be linked functionally in some way. At lower frequencies of stimulation, which produce staircases with small steps, slow contractions take the form of superimposed secondary responses which get bigger as the facilitated response gets smaller (Ross, 1957*a*). In some *Calliactis* sphincter preparations the quick contractions are very weak even at higher frequencies, and usually these are surmounted by immense slow contractions, suggesting that the failure of the quick contraction is linked with enhancement of the slow responses which follow later. It is also noteworthy that Ca^{2+} and tyramine, which selectively augment the quick contraction, tend to depress the slow responses (Fig. 4*b*). All these facts point to a complementary relationship between the two responses of the sphincters, as if some transmitter, produced when impulses reach the muscle, contributes something to any quick contractions that occur, and the remainder (or its product) evokes the later slow contraction. In subsequent work it will be important to look for factors which might explain this complementary relationship between the two responses of the sphincters.

SUMMARY

1. Isolated marginal sphincters of *Calliactis* and *Metridium* show little spontaneous activity, but over a limited frequency range give quick facilitated contractions to not less than two stimuli, and slow contractions having latencies of many seconds to multiple stimuli over a wider frequency range.

2. Earlier observations on the effects of ions on whole animals are confirmed and extended. Excess K^+ causes spontaneous quick contractions and responses to single stimuli; excess Ca^{2+} has no direct excitatory effects, but causes an immense enhancement of the quick response without affecting the slow response to stimulation; excess Mg^{2+} abolishes both quick and slow contractions.

3. Most drugs, including acetylcholine and its associates, histamine, and 5-hydroxytryptamine, have no effects on either quick or slow contractions. Important effects occur only with tyramine, tryptamine and adrenaline at concentrations of 1×10^{-5} and 1×10^{-4}. No inhibitory drug was discovered.

4. Tyramine has no direct excitatory effects but it greatly enhances the quick facilitated responses without enhancing (and in some circumstances depressing) the slow responses to stimuli. It also causes small responses to single stimuli.

5. Tryptamine causes direct quick contractions of the sphincter, gives quick responses to single stimuli and somewhat enhances both quick and slow responses to stimuli.

6. Adrenaline has no effect on quick facilitated responses but causes direct slow responses and some enhancement of slow responses to stimuli.

7. The effects of ions and drugs are obtained only when they are applied directly to the sphincter region. They do not occur when they are applied only to an attached strip of column.

8. It is noted that the effects of excess K^+ and tryptamine are identical, and that the effects of excess Ca^{2+} and tyramine are also identical; adrenaline stands alone as a 'slow-excitor'.

9. The physiological significance of the results is discussed in relation to different components of the responding system and to the mechanisms of quick and slow contraction in the sphincters.

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THE ARGINASE ACTIVITY OF THE TISSUES OF THE EARTHWORMS *LUMBRICUS TERRESTRIS* L., AND *EISENIA FOETIDA* (SAVIGNY)

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Cohen & Lewis (1950) found that the arginase activity of the tissues of *Lumbricus* was related to the output of urea by intact worms, being much higher in fasting than in feeding individuals. This implies that the earthworm may produce excretory urea by the Krebs–Henseleit mechanism (Krebs, 1934) or by some variant of it. In general invertebrates are not ureotelic and it has been suggested (Hunter & Dauphinee, 1925; Baldwin, 1953, pp. 305, 321) that they lack enzymes for the Krebs–Henseleit mechanism. Earthworms therefore are particularly interesting in this connexion.

Living *Eisenia foetida* produce less urea per unit weight than *Lumbricus*, when fasting, and show a smaller change in its output between the fasting and feeding conditions (Needham, 1957), so that the species provides suitably contrasting material on which to extend the experiments of Cohen & Lewis. The arginase activity of homogenates of its tissues, i.e. the rate of liberation of urea from added arginine, has therefore been measured, under fasting and feeding régimes. The tissues of *Lumbricus* have been studied under the same conditions, in order to compare the species differences in arginase activity with those in urea output by the intact worms (Needham, 1957).

Cohen & Lewis found a very high arginase activity in the gut tissue of *Lumbricus* and very low activity in the body wall, or 'carcass', and the two components therefore have been separated for study in the present experiments also.

METHODS

For each experiment a single individual of *Eisenia* was used, or a portion of *Lumbricus* of comparable weight, between 200 and 300 mg. The worms were killed by immersion in 10% ethanol for 5 min. at room temperature. They were freed of superficial fluid and mucus, and weighed. The body wall was opened longitudinally and pinned out. The septa were broken down and coelomic fluid absorbed with filter-paper. The gut was opened along its length and the contents mopped away with damp filter-paper. It was then dissected out from the body wall and both placed on dry filter-paper for 1 min. to remove adherent fluid, before weighing. In fasting *Lumbricus* gut and body wall weighed 12·6 and 48·7%, respectively, of the total wet weight, and 11·1 and 52·9%, respectively, in feeding individuals. For *Eisenia* the four values, in the same order, were 13·4, 53·2, 15·0 and 49·0%. The régime differences are in opposite directions in the two species.

Each tissue was homogenized by hand in a tube of the Potter type. The gut was completely homogenized within 1 min. but the body wall, particularly of *Lumbricus*, required 3 min. before all solid fragments were broken up. Small quantities of earthworm Ringer (Pantin, 1948) were added to the tube, and the suspended homogenate was forced out into a tared tube by the plunger. Grinding and suspension were continued until all of the material had been ejected in this way. 1 ml. of 0·02 M cobalt chloride was added, as co-factor for the arginase, followed by 4 ml. of M/4 disodium hydrogen phosphate as buffer. As antibiotics, streptomycin and penicillin were added in amounts designed to give 1 mg. (735 units) and 0·3 mg. (500 units), respectively, per ml. of the final preparations. The volume was made up to 8 ml., which was thoroughly mixed and divided equally between two incubation tubes, one to serve as control. The tared tube was washed out with 8 ml. of Ringer, shared equally between the two incubation tubes. Finally, 2 ml. of a 2% solution of the substrate, L-arginine monohydrochloride, were added to the experimental tube and well mixed; 2 ml. samples were then withdrawn from both tubes for a zero-hour estimation.

The tubes were lightly stoppered and incubated for 6 hr. at 23° C., the temperature previously used for the study of urea output by intact worms (Needham, 1957). The average pH of control and experimental tubes was 7·8 and 7·6, respectively. The small difference between the two is of little consequence since there was relatively little activity in the control tube. Arginase activity is maximal at a higher pH than this, but destruction of the enzyme also is more rapid.

The urea liberated was estimated as ammonia, after incubating 2 ml. samples for 20 min. at 30° C. with 10 mg. of soya-bean meal as a source of urease. The mixture was well shaken to ensure adequate solution of this enzyme. The resulting ammonia was estimated by the Sørensen formaldehyde method; any ammonium and amino groups present also would be included in the estimate, which therefore may be called the AAU-nitrogen content of the sample. The difference in content between experimental and control tubes may be taken as due to urea-nitrogen released from the added substrate, together with known amounts of amino-nitrogen introduced by the substrate. These are the α-NH_2 nitrogen of the arginine and the ω-NH_2 of the ornithine released, the three guanidino nitrogens of arginine being Sørensen-negative. It is clear that the ornithine ω-NH_2 nitrogen should be exactly one-third of the total nitrogen released from arginine, but that as a check it could be estimated directly, by omitting the urease treatment. This was done on one sample. The zero-hour estimate for the experimental tube, after deducting the control reading, gave a check on the concentration of arginine α-NH_2 nitrogen and was used as the measure of this.

From the results 'specific' values were computed, i.e. the initial content of AAU-nitrogen per unit weight of total nitrogen in the homogenate of the control tube, and the weight of urea nitrogen subsequently released per hour per unit weight of total nitrogen in the experimental tube. Cohen & Lewis (1950) expressed some of their results on the basis of wet weight, and present estimations, on eight samples of each tissue, showed that total nitrogen as a percentage of wet weight varies little, except in fasting *Eisenia*. It was estimated by the Kjeldahl method, using a Markham semi-micro apparatus for the steam-distillation. In the gut and body wall of fasting *Lumbricus* the values were 2·20 and 2·55 % of wet weight, respectively, and 2·16 and 2·32 %, respectively, in feeding individuals. For *Eisenia* the four values in the same order were 3·21, 2·90, 2·43, 2·21 %. The values are seen to be consistently higher in the tissues of fasting worms than in the corresponding tissues of feeding ones, and in those of *Eisenia* than in the corresponding tissues of *Lumbricus*. The gut/body wall ratio was greater than unity in *Eisenia* but less in *Lumbricus*.

In this series of experiments 216 preparations were studied. In addition 164 were used for an earlier series in which thymol was used as antiseptic. The results for that series were in accord with those of the present series but will not be considered in detail.

RESULTS

(1) *Arginase activity*

The results are summarized in Table 1. Those for *Lumbricus* are in close agreement with those of Cohen & Lewis (1950) both in general features and in the actual values for specific arginase activity given in their Table 2. Activity was higher in fasting than in feeding worms; in gut tissue the difference was significant with a value of P considerably less than 0·01. In the body wall activity was low and the régime difference was not statistically significant during this period. Specific activity in the gut was around thirty times that in the body wall. The difference in activity between corresponding tissues of fasting and feeding worms was less than that recorded by Cohen & Lewis but their feeding worms were all direct from the field, whereas those of the present experiments were laboratory-fed for a period of weeks.

In *Eisenia*, also, specific activity was higher in gut than in body wall, and under both régimes the difference was significant beyond the 1 % level. The difference was considerably less than in *Lumbricus*, however. In virtue of its relative bulk the body wall therefore makes a major contribution to the total arginase activity of *Eisenia*.

For both tissues the fasting/feeding difference in specific activity was less than in *Lumbricus* and was not statistically significant. Although more consistently higher in fasting worms when calculated on a wet-weight basis, the difference still was not significant. The régime difference in specific urea output by living worms similarly was found to be less than in *Lumbricus* (Needham, 1957).

The specific activity of the gut of fasting *Lumbricus* was significantly greater than that of *Eisenia*, and the species difference in urea output by living fasting worms must depend mainly on this tissue, since the specific activity of the body wall was significantly higher in *Eisenia*, under both régimes. In feeding, as in fasting, worms the activity of the gut of *Lumbricus* was greater than that of *Eisenia*, but here the significance of the difference was not beyond the 2% level. Allowing for the relative bulk of the body wall, the total activity of the tissues therefore is greater in feeding *Eisenia* than in feeding *Lumbricus* (p. 780), and this is true of urea output by living worms.

(2) *Direct estimation of ornithine released*

For the gut and body wall, respectively, of fasting *Lumbricus* the amounts of ornithine ω-NH_2 nitrogen released were 43·6 and 39·2% of the total Sørensen-nitrogen released from added arginine, and for feeding worms 42·4 and 36·7%, respectively. The four values for *Eisenia*, in the same order, were 38·1, 40·8, 37·8 and 42·1%. These are all greater than the 33·3% expected (p. 776) and for the tissues collectively the excess no doubt is significant. That for the gut of fasting *Lumbricus* gave a value of P less than 0·01 and that for the body wall of feeding *Eisenia* a value between 0·01 and 0·02.

A possible explanation is that the tissues have a slight urease activity. This enzyme is not uncommon in invertebrate tissues (Prosser *et al.* 1950, p. 203; Baldwin, 1953, p. 270). The activity varied between 5 and 17% of the arginase activity of the corresponding tissues, and this relative activity seems reasonable in animals which excrete a substantial proportion of urea as such. The relative activity was sufficiently uniform to give specific activities with much the same tissue, régime and species differences as for arginase activity, which would be consistent with a normal metabolic association between the two.

It is seen that the percentage excess was greater in the body wall than in the gut of *Eisenia*, but conversely in *Lumbricus*. A similar inverse relationship between the two species was seen (p. 777) for the nitrogen contents of the two tissues, the value being relatively low where the present activity was high. No functional association between this activity and low nitrogen content is necessarily implied, though the other species differences encountered in this work did show a different pattern. The species difference in the pattern indicates that the activity is genuine and not due to a technical factor.

(3) *Initial content of AAU-nitrogen*

This includes not only amino + ammonia + urea nitrogen present in, or produced by, the tissues themselves (p. 776), but also some due to the added urease and antibiotics, mainly to streptomycin. These extraneous components were estimated on samples from 'bank' tubes, i.e. control tubes lacking the tissue homogenate, and were deducted from the control tube values for the purpose of this section. The latter were small, and so subject to a considerable percentage error of measurement, but the residual values merit brief consideration. They show features which might justify more specific investigation.

The initial specific content of AAU-nitrogen of the control tubes (Table 1) showed a general proportionality to the arginase activity of the tissue, and was equivalent to about an hour's activity of the enzyme in the experimental tubes. This nitrogen therefore may originate, in part at least, from prior activity of arginase, *in vivo*, or from some correlated activity. Cohen & Lewis (1950) found that the

Table 1. *Mean specific content of ammonia + amino + urea (AAU) nitrogen in fresh homogenates of gut and body wall of* Lumbricus terrestris *and* Eisenia foetida, *in mg. per g. total nitrogen and mean specific arginase activity of the homogenates, in mg. of urea nitrogen released from added arginine per g. total tissue nitrogen, per hour*

Species, régime and tissue	No. of records	Initial AAU-nitrogen content	Arginase activity
		Lumbricus	
Fasting			
Gut	46	175·4 ± 14·9	489·7 ± 56·5
Body wall	20	84·3 ± 6·7	16·3 ± 6·4
Feeding			
Gut	42	160·7 ± 25·1	292·3 ± 50·1
Body wall	20	87·9 ± 13·3	11·8 ± 3·2
		Eisenia	
Fasting			
Gut	24	360·1 ± 68·8	154·3 ± 15·2
Body wall	24	149·4 ± 20·0	67·4 ± 10·1
Feeding			
Gut	20	282·4 ± 39·6	172·9 ± 29·2
Body wall	20	102·0 ± 11·9	38·5 ± 9·7

urea content of the tissues of *Lumbricus* in fact increased, as the enzyme activity does, during fasting. Like arginase activity, AAU-content was significantly higher in the gut than in the body wall of both species, under both régimes. However, the differences were smaller than for arginase activity. Again, in general specific AAU-content was higher in the tissues of fasting worms than in the corresponding tissue of feeding worms, though none of these régime differences was statistically significant. Clearly the correlation is only partial and there were other differences from the pattern of arginase activity: for instance, not only in the body wall but also in the gut it was greater in *Eisenia* than in the corresponding tissue of *Lumbricus*. None of these species differences was statistically significant but this may be due to the large error of measurement and to the difference in one being partly offset by that in another component. The total AAU from the body wall would be greater than that from the gut tissue in both species, under both régimes, whereas for arginase activity this was true only in fasting *Eisenia*. The presumption must be that the initial AAU-content reflects intermediate nitrogen metabolism rather more generally.

DISCUSSION

The present results for *Lumbricus* confirm those of Cohen & Lewis (1950), while those for *Eisenia* further strengthen their conclusion that the arginase activity of the tissues of earthworms is related to the excretion of urea by living worms. It has been seen that the relationship is manifested in a number of further details. Ideally arginase activity should be measured in living worms, or spontaneous urea excretion in tissue homogenates, in order to make a critical comparison between them. It is possible, from the present results, to compute a total arginase activity, per gramme of intact worm, but this ignores any possible activity of gut contents or of body fluid and also any systematic control of enzyme activity, and it would not be surprising if the results did not agree closely with figures for urea output by the corresponding living worm. The values obtained were 156, 85, 170 and 105 mg. of urea nitrogen per hour per 100 mg. wet weight of whole worm, respectively, for fasting and feeding *Lumbricus*, and fasting and feeding *Eisenia*. Apart from that for fasting *Lumbricus*, the figures in fact bear similar proportions to the corresponding urea outputs by living worms, 129, 41, 73 and 48 mg. of urea nitrogen per gramme per day (Needham, 1957). The particularly high output of urea by living fasting *Lumbricus* therefore may depend as much on systemic factors as on the high intrinsic activity of arginase in its gut tissue. There is in fact evidence for a systemic control of urea output (Needham, 1958).

Cohen & Lewis (1949) found also that the feeding of arginine to *Lumbricus*, and to a lesser extent that of citrulline, caused an increase in urea excretion, so that the enzyme presumably does function in normal urea excretion, *in vivo*. When the common amino acids were fed along with citrulline urea output was increased more than by citrulline alone and this probably implies that part of the urea, at least, comes from the deamination of amino acids in general. Abdel Fattah (1954) obtained an increased urea excretion by feeding the common amino acids alone. Cohen & Lewis failed to demonstrate this, perhaps because their animals had insufficient indigenous citrulline, but there is also the complication that, when feeding, *Lumbricus* excretes mainly ammonia and not urea, so that the former is probably the final product of deamination of any amino acids given as food. Possibly for the same reason they found that ornithine fed together with other amino acids likewise caused no increase in urea excretion. These authors further found that ornithine alone, in contrast to citrulline alone, caused no increase. This may indicate that, in the control of urea excretion, arginine synthesis may be halted at the ornithine, but not at the later, citrulline stage.

The arginase activity found in the body wall of *Lumbricus* is low enough to prompt the suggestion of Cohen & Lewis that it is due merely to contamination from the gut during dissection. However, this cannot be true in *Eisenia* where the total activity measured in the body wall is virtually equal to that of the gut; the activity associated with the body wall therefore may be intrinsic in *Lumbricus* also. Much of the nephridial tissue remains attached to the body wall and may be responsible for the activity measured. If so, then there is an anatomical analogy with the vertebrates, of which the kidney tissue similarly possesses some arginase activity, and the specific activity is less than that of the liver (Hunter & Dauphinee, 1925), which is a gut appendage. The chloragogen tissue of earthworms has some resemblance to the digestive gland tissue of Crustacea and Mollusca, and Abdel Fattah found a particularly high urea content and arginase activity in the chloragogen tissue.

There are general grounds, as well as some evidence from the present results, for believing that much of the AAU-nitrogen originally present in the homogenates is related to the excretion of the three components by the living animal. This also can be tested further by computing the corresponding values for whole worms and comparing these with the AAU-excretion previously recorded for living worms (Needham, 1958). The values obtained, 153, 146, 385 and 213 μg. per 100 mg. wet weight of whole worm, respectively, of fasting and feeding *Lumbricus* and fasting and feeding *Eisenia*, are very near to the amounts of AAU-nitrogen excreted per gramme per day by living worms, viz. 159, 95, 346 and 205 μg. The largest percentage discrepancy is that for feeding *Lumbricus*, which therefore appears to excrete less of the AA-components than indicated by the activity of its tissues (the urea component was shown above to agree with expectation, in contrast to that of fasting *Lumbricus*). Like those for AAU-excretion by the living worm, the values are considerably higher in *Eisenia* than in *Lumbricus* and for both properties the fasting/feeding ratio shows a comparable difference between the two species.

The conclusion that the initial AAU-content is related to normal nitrogen excretion could be tested by specific experiments designed to measure the ammonia-producing activity of the tissues, for instance, the glutaminase I activity, using glutamine as substrate, and the proteolytic activity, using a suitable protein as substrate.

The properties studied are seen to vary less between tissues and régimes in *Eisenia* than in *Lumbricus*. The latter therefore is probably the more highly evolved genus.

It is interesting that the vertebrates, which as a group are ureotelic (Brown & Cohen, 1960), use creatine phosphate and not arginine phosphate as the phosphagen of their muscles and that earthworms also possess a phosphagen, lombricine (Rosenberg & Ennor, 1959), which is not based on arginine. There is a possibility, therefore, that muscle arginine might be excessively destroyed in ureotelic animals, which in consequence have exploited a different phosphagen. Possible objections to this view are that little arginase is usually present in muscle tissue and that molluscs, such as *Helix*, appear to excrete considerable urea but yet to use arginine phosphate as their phosphagen.

SUMMARY

1. The difference in arginase activity between the tissues of *Eisenia* and those of *Lumbricus* shows a relationship to the difference in urea output by living worms of the two species under the same dietary régime.

2. In *Eisenia* the difference in activity between the tissues of fasting and feeding worms is much smaller than in *Lumbricus*. The specific outputs of urea by living, fasting and feeding worms likewise differ less than in *Lumbricus*.

3. These facts strengthen previous evidence in favour of a Krebs–Henseleit type of mechanism for urea production in earthworms.

4. In *Eisenia* the difference in arginase activity between gut and body wall is similar to, but smaller than, that in *Lumbricus*, and the body wall makes a major contribution to the total activity.

5. The combined concentrations of ammonia-, amino-, and urea-nitrogen initially present in homogenates of the tissues of these worms are proportional to

the combined amounts of the three components excreted per unit weight by living worms of the same species and régime.

6. The two species differ in a number of other properties investigated.

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EXPERIMENTS ON THE INFLUENCE OF LIGHT ON THE BEHAVIOUR OF POLYZOAN LARVAE

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INTRODUCTION

The majority of marine polyzoans have a larva which hatches at an advanced stage of development and settles soon after liberation. Throughout its short pelagic life the behaviour of the larva is strongly influenced by light. In fact, the hatching process itself appears to occur in response to illumination in *Bugula simplex* (Grave, 1930), *B. neritina* (Lynch, 1947) and *B. turrita* (Lynch, 1949). (The nomenclature of the various species of *Bugula* has been corrected where necessary, and follows the account of Ryland (1960).) In all other species investigated during the present work also, light was found to be the stimulus required to initiate hatching. It should be noted, however, that all the present, and previously published, observations refer to species found in shallow water or on the shore; there are no data referring to deep-water species.

Two related but distinct types of behaviour will be discussed. First, the responses of larvae to directional illumination during the free-swimming phase and, secondly, the effect of illumination on the settlement behaviour. The experiments appropriate to each will be described and discussed separately.

REACTIONS OF LARVAE TO LIGHT DURING THE FREE-SWIMMING PHASE

It is well known that the behaviour of many marine larvae and other planktonic organisms is strongly influenced by light. These may display markedly photopositive or photonegative reactions, and Spooner (1933) has shown that the response is to the direction rather than the intensity of illumination. It also seems probable that the responses of polyzoan larvae to light are accompanied by responses to gravity. Thus, when first liberated, many larvae, such as those of *Celleporella hyalina* and *Bowerbankia imbricata*, accumulate near the surface of the water on the illuminated side of the container. Grave (1930) and Lynch (1947) make similar observations with reference to other species.

Previous work on light as a factor influencing larval behaviour has sometimes suffered from certain defects. First, the conditions of illumination have been badly defined, making it difficult for other workers to repeat them with accuracy. A second criticism is that the light intensity employed was usually very low and has borne little relation to that existing normally in the surface waters of the sea. In summer, at latitude 50° N. with the sun at zenith and shining, for example, the illumination at the surface might amount to 120 kilolux or more, of which some 4–25 % would be reflected according to the state of the surface. At a depth of 10 m. in average coastal water the illumination would be in the region of 6800 lux, and about 2600 lux in turbid water (combined from Sverdrup, Johnson & Fleming, 1942; and Harvey, 1955).

Thirdly, no attempt has been made to describe the behaviour in quantitative terms. A method was required, therefore, which did not suffer from these three defects, and would permit the investigation of the influence of directional illumination unaffected by any possible effects due to gravity.

Apparatus

The apparatus which was designed for these experiments, somewhat modified in the light of later experience, is shown in Fig. 1. The light source employed was a theatre spot-lamp fitted with a 500 W. tungsten filament bulb. The lamp had a silvered reflector and an adjustable front lens which permitted focusing of the light beam. The lamp provided a virtually parallel beam of considerable intensity. In all the experiments described below, the illumination employed was 5000 lux, which is considerably higher than has generally been used for studying the reactions of marine organisms to light. Since most polyzoans breed in the latter part of the summer, this intensity was considered a suitable one (see above), and it also showed up the larvae well.

The intensity could be reduced by inserting neutral density filters. (A rheostat is unsuitable for this purpose as it would alter the colour temperature.) The spectral composition of the light could also be altered by the use of colour filters, notably the Ilford 810 which can be used with a tungsten source to provide light approximating closely in quality to mean noon daylight. A mask in front of the lamphouse reduced the size of the beam, while another was placed in front of the apparatus to exclude stray light. A long distance between the lamp and the troughs is an important feature essential for a well-collimated beam.

The experimental vessels in which the larvae were placed were rectangular Perspex troughs, 20 × 2 × 2 cm., whose long axes were orientated in the plane of the light beam. The first set consisted of six parallel troughs, but it was later found desirable to have two trough units which could be used independently. Each unit consisted of a pair of troughs either one of which acted as control for the other. (While the experiments described in this paper were conducted in rectangular troughs, the apparatus now in use incorporates the paired U-shaped trough units shown in Fig. 1.)

The troughs stood over a black card divided into ten 2 cm. divisions by thin white lines. These were numbered I–X, but in some of the earliest experiments a background divided into eight divisions was used. When the light was projected along the troughs, the larvae stood out clearly against the black background, and it was possible to record the number of larvae above each of the divisions marked on the card. The larvae were most difficult to count against the trough end-walls, partly

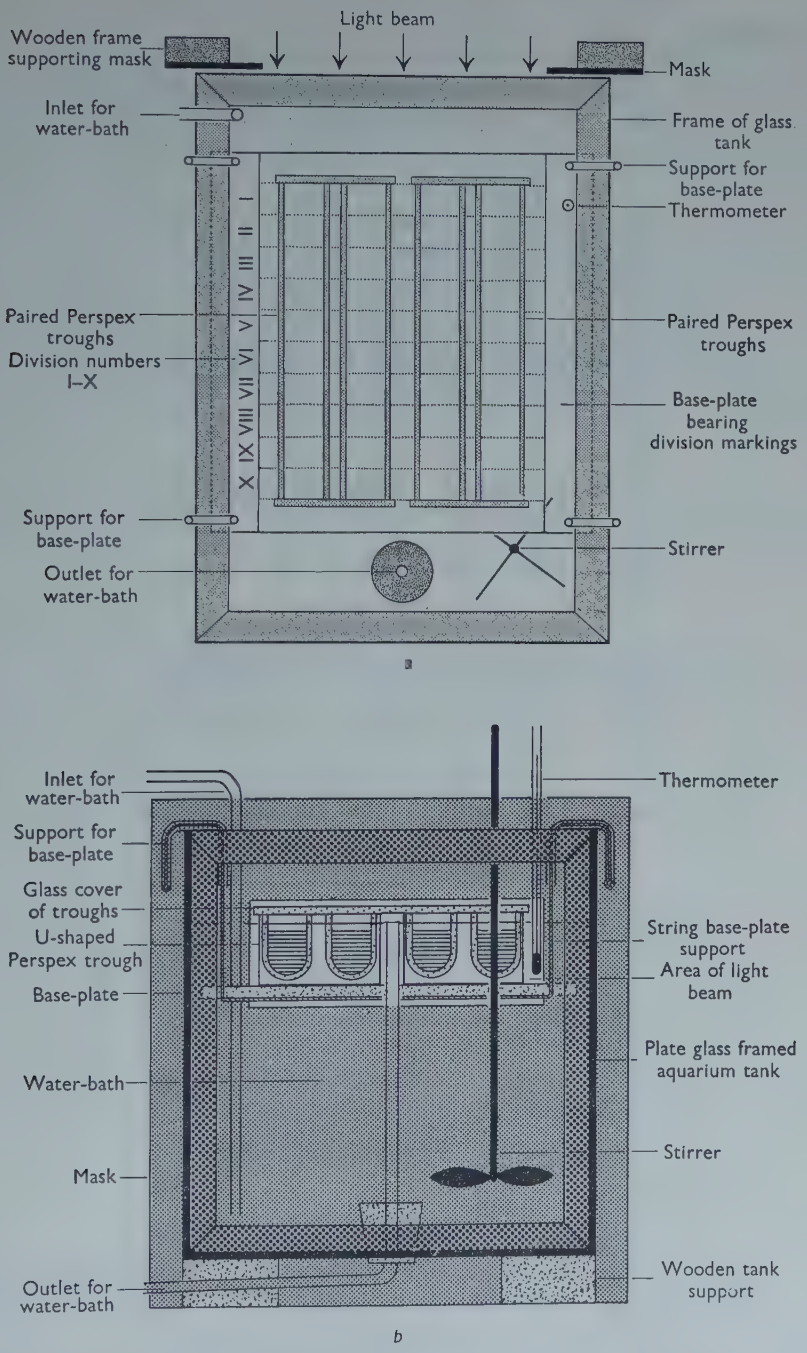


Fig. 1. Apparatus used for experiments investigating the responses of marine larvae to directional illumination. (*a*) Seen from above. (*b*) End view, looking towards the light source. For full description see text.

because numbers were usually highest at one or other of the ends, and partly because of the presence of corners which seemed to attract the larvae. The first difficulty was not serious if only about twenty larvae were used per trough. The use of troughs of U-shaped section eliminates the corners, and these also have the added advantage of a larger and more intimate contact with the water in the constant temperature bath.

The set of six troughs, or the trough units, stood on a glass base-plate supported in an aquarium tank filled to just above trough water level to maintain a constant temperature. The water-bath incorporated a thermometer at trough level and an electric stirrer (or heater-stirrer in experiments with *Bugula neritina*). Fresh cold water could be added if required, and the requisite level was maintained by a siphoning device. Ordinary moulded glass tanks have been used for the water bath, but the framed type with plane plate-glass walls, now being used, is to be preferred.

The trough units were covered by a sheet of glass. This helped to prevent their being moved by agitation of the water in the tank, kept the trough water free from dust, and reduced evaporation.

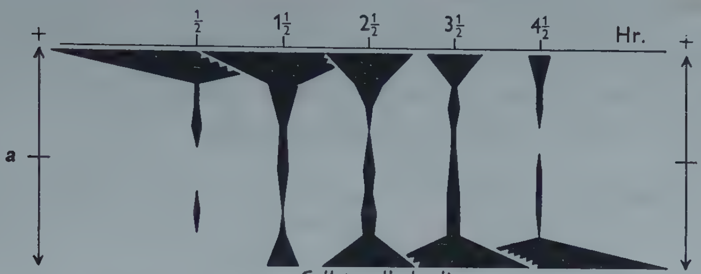
Experimental method

The polyzoan colonies were kept in running sea water in total darkness. When larvae were required for experiments, the colonies were illuminated by tungsten or daylight. Wherever possible, as with the tufted colonies of species of *Bugula*, the zoaria were placed in the troughs so that the larvae were liberated *in situ*. With incrusting species it was necessary to keep large quantities of polyzoan-covered alga in tanks, and pipette the larvae gently into the troughs. Pipetting, even when carried out gently with a wide-mouthed pipette, sometimes influenced the behaviour of the larvae. In *B. neritina* and *Escharoides coccineus* some of the larvae reacted by immediately metamorphosing on the surface film, while in other species a few larvae changed the sign of their reaction to light and became prematurely photonegative. When this happened the experiment had to be restarted with fresh larvae.

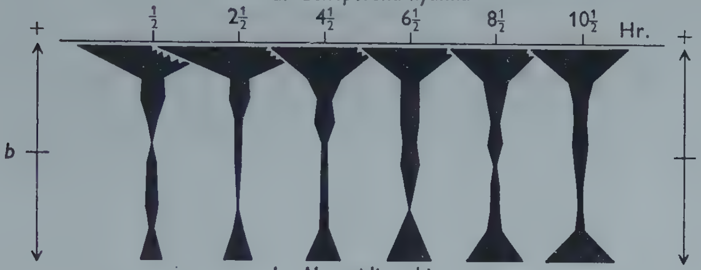
The water in the troughs was filtered to remove planktonic organisms, and prior to the experiment was stored in a container placed, with the troughs, in the tank containing the polyzoan colonies. In this way it was possible to avoid subjecting the larvae to abrupt changes of temperature. The apparatus was left for about $\frac{1}{4}$ hr. before the first reading was taken. Then and at regular intervals the positions of the larvae were recorded.

Results and discussion

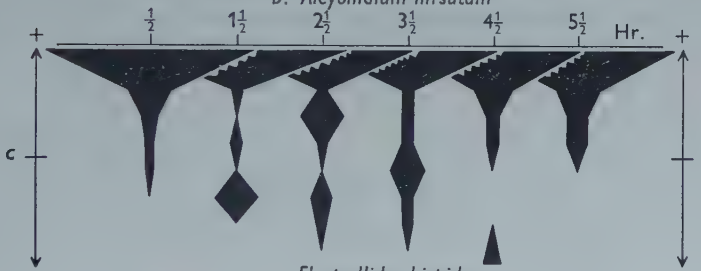
The behaviour of the larvae investigated in the troughs conformed to one of four patterns (Tables 1–9; Fig. 2) referred to as types I, II, III and IV. In type I (Fig. 2*a*) the larvae were at first photopositive, almost the whole population swimming against the end-wall nearer the light source. After the lapse of an hour or two the population as a whole tended to become photonegative and eventually became almost completely so. Since at any moment larvae could be seen swimming along the troughs in both directions, there was no definite and clear-cut reversal of



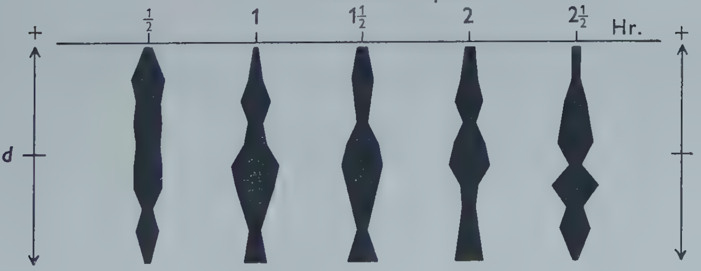
a. *Celleporella hyalina*



b. *Alcyonidium hirsutum*



c. *Flustrellidra hispida*



d. *Alcyonidium polyoum*

Fig. 2. The responses of polyzoan larvae to directional illumination. The kite diagrams show the behaviour of larvae of four species in troughs illuminated from one end. The troughs were marked into divisions and the distribution of larvae plotted at regular intervals. Four types of behaviour were recorded, and one example of each is figured. (*a*) Type I: *Celleporella hyalina* (Table 1). The larvae were photopositive at first, but became negative after a few hours. (*b*) Type II: *Alcyonidium hirsutum* (Table 6). The larvae were all photopositive at first, and the majority remained so. (*c*) Type III: *Flustrellidra hispida* (Table 7). The larvae remained photopositive, even when the experiment was continued for several hours. (*d*) Type IV: *Alcyonidium polyoum* (Table 9). The larvae remained generally distributed in the troughs, and were neither positive nor negative to light.

behaviour, but rather a trend shown by the population. Some species which behave in this way are *Celleporella hyalina*, *Bugula fulva*, *B. plumosa*, *Scrupocellaria scruposa* and, also, the serpulid worm *Spirorbis borealis* (Tables 1–5). A similar reversal has been noted in the polyzoans *Bugula simplex* and *B. turrita* (Grave, 1930; Lynch, 1949) and *Scrupocellaria reptans* (Lutaud, 1953).

Table 1. *The behaviour of* Celleporella hyalina *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 17° C. (see text-fig. 2a). The larvae were liberated in the troughs*

Time (hr.)	Numbers of larvae									
	Photopositive								Photonegative	
	I	II	III	IV	V	VI	VII	VIII	IX	X
½	45	1	1	2	0	0	0	1	0	0
1½	33	9	6	2	2	3	1	0	2	7
2½	18	7	2	0	1	3	2	4	3	18
3½	10	1	3	1	1	2	3	3	3	28
4½	3	1	1	0	0	1	1	1	0	34

Table 2. *The behaviour of* Bugula fulva *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 15·5° C. The larvae were liberated in the troughs*

Time (hr.)	Numbers of larvae									
	Photopositive								Photonegative	
	I	II	III	IV	V	VI	VII	VIII	IX	X
½	19	11	22	7	3	0	0	1	0	7
1½	13	7	3	6	2	1	1	3	2	11
2½	5	2	0	0	2	1	0	4	3	21
3½	10	3	3	1	1	1	3	2	5	34
4½	6	3	2	1	1	1	5	5	4	38

Table 3. *The behaviour of* Bugula plumosa *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 16·25° C. The larvae were liberated in the troughs*

Time (hr.)	Numbers of larvae									
	Photopositive								Photonegative	
	I	II	III	IV	V	VI	VII	VIII	IX	X
½	51	10	5	5	0	0	0	0	0	0
1½	22	9	9	9	3	5	1	1	0	9
2½	11	3	0	1	0	1	3	4	6	33

Larvae showing type II behaviour tended to remain photopositive, although a proportion eventually swam away from light. This type of behaviour was most clearly shown by *Alcyonidium hirsutum* (Table 6, Fig. 2b). Type III behaviour, in which the larvae remained photopositive throughout the experiment, was clearly shown by *Flustrellidra hispida* (Table 7, Fig. 2c).

Bugula neritina larvae were found to belong to type II (Table 8). Many of the larvae metamorphosed, the highest settlement occurring at the end of the trough toward the source of illumination, but there was a definite, though partial, swing

Table 4. *The behaviour of* Scrupocellaria scruposa *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 15° C. The larvae were liberated in the troughs*

Time (hr.)	Numbers of larvae							
	Photopositive						Photonegative	
	I	II	III	IV	V	VI	VII	VIII
½	17	2	1	0	1	0	1	3
1	10	0	0	0	1	1	2	10
1½	4	0	1	1	0	1	4	9
2	4	0	1	0	0	0	2	12
2½	1	0	0	0	1	1	0	14

Table 5. *The behaviour of* Spirorbis borealis *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 15° C. The larvae were transferred to the troughs with a pipette*

Time (hr.)	Numbers of larvae							
	Photopositive						Photonegative	
	I	II	III	IV	V	VI	VII	VIII
¼	62	8	9	12	6	8	2	2
1	62	7	0	8	10	6	4	13
2	59	5	2	5	3	3	6	17
3	35	8	7	6	6	11	9	27
4	8	2	4	4	8	10	10	45
5	5	2	3	5	4	2	10	66

Table 6. *The behaviour of* Alcyonidium hirsutum *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 8·5° C. (see text-fig. 2b). The larvae were transferred to the troughs with a pipette*

Time (hr.)	Numbers of larvae									
	Photopositive								Photonegative	
	I	II	III	IV	V	VI	VII	VIII	IX	X
½	49	10	11	4	1	4	4	5	1	7
2½	59	8	4	0	2	0	3	3	2	14
4½	37	14	6	8	2	3	3	3	4	11
6½	33	8	7	7	6	7	3	1	7	16
8½	30	12	8	4	6	1	4	2	6	20
10½	33	10	6	5	6	4	3	1	3	22

Table 7. *The behaviour of* Flustrellidra hispida *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 15° C. (see text-fig. 2c). The larvae were transferred to the troughs with a pipette*

Time (hr.)	Numbers of larvae							
	Photopositive						Photonegative	
	I	II	III	IV	V	VI	VII	VIII
½	18	5	2	1	1	0	0	0
1½	15	1	0	1	0	4	0	0
2½	13	1	4	1	0	2	1	0
3½	13	1	1	1	3	1	1	0
4½	11	3	1	1	0	0	0	1
5½	7	2	1	1	0	0	0	0

Table 8. *The behaviour of* Bugula neritina *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 24° C. The larvae were transferred to the troughs with a pipette*

	Numbers of larvae									
Time (hr.)	Photopositive I	II	III	IV	V	VI	VII	VIII	IX	Photonegative X
½	74	0	0	0	0	0	0	0	0	1
1½	59	2	2	0	2	1	3	0	2	4
2½	52	0	1	1	0	1	0	2	1	17
3½	51	1	0	0	0	0	2	1	2	18

to photonegative behaviour. Both McDougall (1943) and Lynch (1947) found that the larvae remained photopositive throughout their free-swimming phase, but Mawatari (1951) recorded a reversal of response. In other experiments Lynch considered that the larvae became 'more or less indifferent to light'. Table 8 shows, on the contrary, that the larvae were showing either a positive or a negative response: if the larvae were indifferent the distribution in the troughs would follow a random pattern.

Lynch (1947) considered that in his experiments the failure of *B. neritina* larvae to become photonegative was due to the low intensity of illumination employed, although, in the same paper, he failed to induce any change by increasing the intensity. Both he and McDougall (1943) thought that in their natural environment the larvae must become photonegative. This seems probable; but McDougall's demonstration that the larvae preferred to settle in a region of low light intensity does not necessarily imply a negative phototaxis. Further work seems desirable to try to relate the behaviour of these and other type II larvae in the laboratory to their behaviour in the sea.

Nitsche (1870) found that *B. flabellata* larvae metamorphosed on the illuminated side of glass containers, but preliminary observations suggest that they may sometimes become photonegative.

Whatever their subsequent behaviour, immediately after liberation the larvae of the great majority of polyzoans are positive to light. In addition to the species already mentioned this has been found true in *Cryptosula pallasiana. Microporella ciliata*, *Fenestrulina malusi*, *Escharoides coccineus* and *Bowerbankia imbricata*, while Mawatari (1952) made the same observation for *Watersipora cucullata*. *Alcyonidium polyoum* seems exceptional in that the behaviour of the larvae appeared uninfluenced by light (Table 9, Fig. 2*d*)—a result which confirms the earlier observation of Zschiesche (1909). This has been called type IV.

The experiments were generally performed at a temperature about equal to that of the sea surface at that time. It is possible that larval responses to light may be influenced by temperature. In *Bugula neritina* a decrease in temperature prolongs the natant stage (Lynch, 1947). Gravity responses of polyzoan larvae are also affected by temperature (Lynch, 1947). This subject is being investigated at the present time.

Table 9. *The behaviour of* Alcyonidium polyoum *larvae in troughs illuminated from one end by a light beam of 5000 lux intensity at a temperature of 17·5° C. (see text-fig. 2d). The larvae were transferred to the troughs with a pipette*

Time (hr.)	Numbers of larvae Photopositive I	II	III	IV	V	VI	VII	VIII	Photonegative IX	X
½	4	9	7	8	7	8	7	3	6	3
1	2	4	8	4	6	13	11	7	3	5
1½	4	6	5	3	9	12	9	6	3	7
2	3	5	7	4	9	11	5	4	5	7
2½	2	2	5	7	8	3	11	4	7	3

Table 10. *Results of an experiment in which one trough unit, A, containing* Celleporella hyalina *larvae was placed in a beam of light of 5000 lux intensity, while a similar unit, B, was kept in complete negative to light, unit B was placed beside it in the light beam. The experiment was conducted at 20° C.*

Time (hr.)	Unit		Numbers of larvae Photopositive I	II	III	IV	V	VI	VII	VIII	Photonegative IX	X
¼	A	i	60	3	5	2	3	5	1	1	1	4
		ii	60	7	10	6	4	4	3	3	1	0
2	B	Placed in light beam	—	—	—	—	—	—	—	—	—	—
2¼	A	i	1	0	2	3	4	2	4	3	14	40
		ii	7	1	0	1	0	0	1	5	10	55
	B	i	2	1	1	1	0	2	4	3	1	32
		ii	1	1	0	0	0	2	2	3	8	36
4¼	A	i	0	1	1	0	1	0	1	1	5	49
		ii	1	0	0	0	2	1	2	3	6	55
	B	i	0	0	0	1	1	0	0	1	1	23
		ii	0	0	0	0	0	0	3	3	10	34

It seems probable that the majority of polyzoan larvae show type I behaviour, being photopositive when liberated, but later swimming away from light. It was clearly of importance to ascertain the cause of this change in behaviour: was it purely a question of time, or the result of a certain amount of light energy being received by the larvae? The observation of Lynch (1947) that *B. neritina* larvae 'swim away from regions receiving the direct rays of the sun after a certain amount of exposure to direct light' appears to imply the latter. To decide the question, two paired trough units were used. Approximately equal numbers of freshly liberated, photopositive larvae of *Celleporella hyalina* were pipetted into each. One unit was placed in the light beam while the other was kept in the dark. When the majority of larvae in the illuminated troughs had become photonegative, the second unit was placed in the light beam beside the first. The results are given in Table 10, and show that, in this species at least, the reversal of behaviour is dependent on the passage of time and not on illumination.

These experiments raise a second problem. To what extent is the reversal of the reaction to light related to the normal settlement of the larvae? Previous

Table 11. *The settlement of* Celleporella hyalina *larvae on disks of* Fucus serratus *in troughs illuminated from one end*

Trough division	Pieces of *Fucus*	Trough						Total
		a	*b*	*c*	*d*	*e*	*f*	
Photopositive								
I	α	1	3	0	3	6	1	14
II								
III	β	2	0	0	1	2	4	9
IV								
V	γ	0	0	0	6	0	0	6
VI								
VII	δ	2	1	1	5	7	2	18
VIII								
IX	ε	16	3	9	13	16	12	69
X								
Photonegative								

experiments had already shown that illumination affected settlement of *C. hyalina* larvae on algal substrates (Ryland, 1959). In another experiment with *C. hyalina*, similar disks cut from *Fucus serratus* fronds were placed at intervals along the length of troughs containing photopositive larvae. The results (Table 11) show that the greatest numbers settled furthest from the light source; it is therefore quite clear that the majority of larvae had developed a photonegative reaction before they settled.

This problem deserves further attention, for it seems desirable to try to relate this pattern of settlement to the amount of exploratory behaviour shown by the larvae. Observation suggests that larvae whose reaction has become photonegative 'explore' more than those still positive to light. That is to say, larvae with a negative reaction do not swim against the end wall so persistently as those which are photopositive. This was also implied by Lynch (1947) who said that the photonegative response 'is never so intense as the positive one'. The results of the experiment just discussed indicate that the pattern of settlement may well be related to the amount of 'exploration'.

THE INFLUENCE OF LIGHT ON SETTLEMENT BEHAVIOUR

The influence of surface angle on settlement: experiments in the sea

The influence of surface angle on the settlement of marine larvae has received considerable attention, notably in relation to the cultivation of oysters. The settlement of organisms has been recorded on panels of various kinds and in different orientations in the sea. The results of earlier workers which related to shell-fish culture were reviewed by Cole & Knight-Jones (1949), who also discussed their own observations. The results of Pomerat & Reiner (1942) and of Maturo (1959) are of special interest since Polyzoa figured largely in their studies. The practice, now conventional, of indicating the orientation of a surface is as follows:

0°	Lower surface of horizontal panels
45°	Lower surface of oblique panels
90°	Vertical surfaces
135°	Upper surface of oblique panels
180°	Upper surface of horizontal panels

It has been recorded by Pomerat & Reiner (1942) and Maturo (1959) that abundant settlement of the polyzoans *Acanthodesia tenuis*, *Bugula neritina*, *Electra hastingsae* and *Schizoporella unicornis* only occurred on the 0° and 45° surfaces. Knight-Jones (1951) found that '*Membranipora lacroixi*'* settled more abundantly on the lower surfaces of horizontally placed slates. Pomerat & Reiner found that the settlement of bivalves was markedly heavier on the 0° surface, and this has been confirmed by Cole & Knight-Jones (1949) for *Ostrea edulis*—although both they and Korringa (1941) occasionally obtained anomalous results in which spatfall was higher on the upper surfaces. Many other animals, but not all, show a preference for lower surfaces (Knight-Jones, 1951).

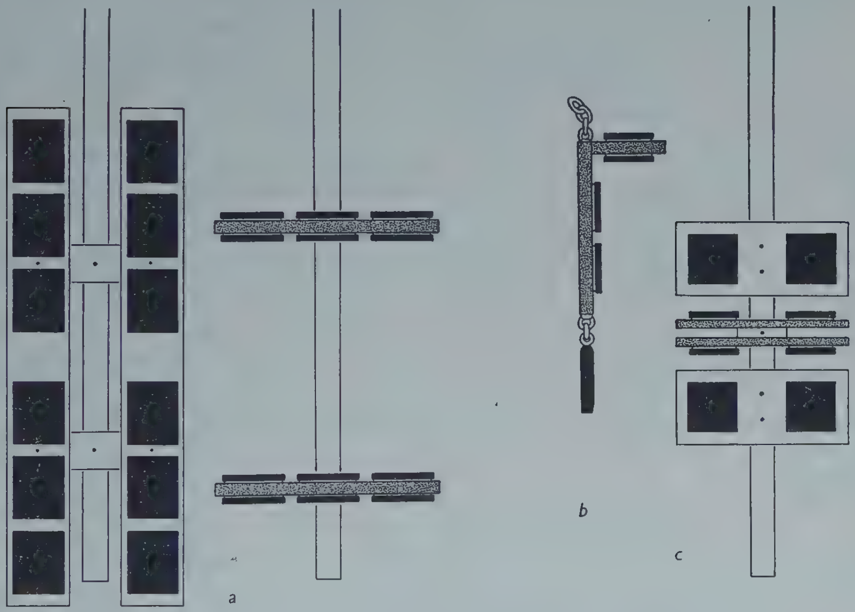


Fig. 3. Collectors used for settlement studies in the Menai Straits. (*a*) Sets of twelve 10 × 12·5 cm. panels used at location A in 1957. (*b*) Set of eight 10 × 10 cm. panels mounted on swivel device (see text) at location B in 1958. (*c*) Set of eight 10 × 10 cm. panels used at location A in 1958. In all cases the panels were mounted with a space of 1 mm. between them and the base-plate.

In the present studies panels fixed on various types of collector were immersed in the Menai Straits with the principal objective of determining the settlement seasons of the commoner Polyzoa. In view of the preference for lower horizontal surfaces reported in the literature, some of the panels were placed vertically and some horizontally (Fig. 3). In fixing the panels a space of 1–2 mm. was left between the panels and the base-plate by inserting washers around the attaching bolts: thus every panel had an outside and an inside surface available for settlement. At locality A, during 1957 and 1958, panels were attached on poles mounted below a raft moored in the Menai Straits. In 1958 a second set was mounted on the swivel device described by Crisp & Stubbings (1957) and located between the floating pontoons of Menai Bridge pier (locality B). In both cases, therefore, the orientation of the panels would follow the direction of the current, while they would also remain at a constant (2–4 ft.) depth below the surface. The settlement recorded on each of the various surfaces is shown in Table 12.

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* Probably included at least two species.

The conditions at A, under the raft, differed in one important respect from those obtaining under the pier (B): the greater amount of illumination allowed the formation of a dense carpet of algae (*Ectocarpus* and *Pilayella*) on the panels during the

Table 12. *The settlement of* Polyzoa *per* 1000 *cm.*^{2} *of surface on the variously orientated panels illustrated in fig.* 3. (*For further explanation see text*)

	Station I, 1957 (Fig. 3*a*)											
	Vertical series				Horizontal series							
	Upper set		Lower set		Upper set				Lower set			
Panel orientation ...	—		—		Above base-plate		Below base-plate		Above base-plate		Below base-pla	
Panel surface ...	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside
Species												
Crisiidae	—	—	1	—	3	—	3	3	—	—	—	—
Callopora dumerili	—	—	—	—	—	—	—	—	—	—	—	—
Electra pilosa	11	2	5	15	16	19	21	119	19	56	5	22
Bugula fulva	—	—	—	—	—	—	—	—	—	—	—	—
Bugula plumosa	—	—	—	—	—	—	—	—	—	—	—	—
Scrupocellaria scruposa	—	—	—	—	—	—	—	—	—	—	—	—
Celleporella hyalina	70	19	43	41	171	5	32	221	136	64	35	8
Cryptosula pallasiana	3	1	2	5	3	—	—	13	—	3	3	
Bowerbankia gracilis	—	—	—	—	—	—	—	—	—	—	—	—
Others	3	1	6	2	11	—	—	11	13	5	5	—
Total	87	23	57	63	204	24	56	367	168	128	48	12

	Station I, 1958 (Fig. 3*c*)						Station II, 1958 (Fig. 3*b*)					
	Vertical series		Horizontal series				Vertical series		Horizontal series			
Panel orientation ...	—		Above base-plate		Below base-plate		—		Above base-plate		Below base-pla	
Panel surface ...	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside
Species												
Crisiidae	3	—	5	—	—	—	5	15	5	45	5	11
Callopora dumerili	—	—	—	—	—	—	63	417	70	15	10	26
Electra pilosa	28	25	5	5	15	20	13	45	15	30	25	19
Bugula fulva	—	—	—	—	—	—	—	145	15	—	—	12
Bugula plumosa	—	—	—	—	—	—	—	18	—	—	—	2
Scrupocellaria scruposa	—	—	—	—	—	—	23	100	15	10	5	10
Celleporella hyalina	30	8	35	5	5	10	10	90	20	5	—	20
Cryptosula pallasiana	—	—	—	—	—	—	5	30	10	—	10	[illegible]
Bowerbankia gracilis	—	—	—	—	—	—	43	98	30	65	60	9
Others	5	—	15	—	—	—	5	25	20	—	10	2
Total	66	33	60	10	20	30	167	983	200	170	125	111

summer months. The maximum settlement of some polyzoans, notably *Celleporella hyalina*, coincided with the period of greatest development of this algal cover. It is not surprising that the presence of this weed inhibited the settlement of polyzoans. Thus at A in 1957 the upper set of vertical panels, 1–2 ft. below the water surface, supported a dense algal growth; consequently the majority of polyzoans settled on the inside surfaces of the panels. On the lower set, 3–4 ft. below the surface, algal growth was sparse, and the settlement of *Celleporella* was about equal on the inside and outside surfaces (Table 12).

The horizontal surfaces showed even more striking differences. The general picture was one of high settlement on surfaces which face downward, and of low settlement on those facing upward.

No algae developed on the panels at B, although, as at A, sediment collected on upward-facing (180°) surfaces.

The data from both localities differ from those previously published in that vertical (90°) surfaces were as favourable, or even more so for some species, than the 0° surfaces. It is clear, however, that those surfaces on which algae grow densely and/or collect sediment do not receive a high settlement of polyzoans, and Maturo (1959) considered silting of the 135° and 180° surfaces to be the major factor preventing their establishment. But this is not necessarily the only explanation of the results; there is another, although its effect may have been masked by the presence of algae plus sediment. The settlement pattern may in fact reflect the general swimming and searching behaviour of the larvae, and some previous workers (e.g. Cole & Knight-Jones, 1949) have explained their results in this way. Since this could only be decided by direct observation in the absence of sediment, further investigations were pursued in the laboratory.

The influence of surface angle on settlement: laboratory experiments

Experiments have been conducted with *Celleporella hyalina* larvae, using filmed opaque (Tufnol) or transparent (Perspex) panels placed vertically, obliquely and horizontally in aquarium tanks. Each of the panels was suspended from supports above the tank by threads attached to the corners. Three panels were used simultaneously so that, in each tank, six surfaces were offered, orientated at 0°, 45°, 90° (two), 135° and 180°. The result given for 90° is, therefore, the mean of the settlement recorded on the two vertical surfaces.

The results of these experiments are shown in Table 13. Since the actual number of larvae that settled in any experiment varied so much, the results are expressed as percentages before being converted to means. A mean based on numbers of larvae set could be somewhat misleading, since the results would be unduly weighted by those experiments in which settlement was very high. The percentage of the total number of larvae set on each of the surfaces is, however, also given in Table 13, but it was not used in the construction of Fig. 4.

Lighting conditions were varied. In some tanks diffuse light from a 100 W. bulb could fall on the panels only from above. In others the light from a similar bulb was reflected by a mirror, through a diffusing screen, from below the panels, the tank

Table 13. *Settlement of* Celleporella hyalina *larvae on opaque and transparent panels orientated in various planes. Conventions are explained in the text*

Expt.	Experimental conditions	Percentage settlement on each of five differently orientated surfaces					Actual no. of larvae set
		0°	45°	90°	135°	180°	
1	Opaque panels (Tufnol) illuminated from above	24·2	54·6	15·1	0·0	6·1	16·5
2		83·9	3·8	4·7	1·9	5·7	52·5
3		64·0	8·0	4·0	24·0	0·0	12·5
4		83·9	6·4	3·3	6·4	0·0	15·5
5		80·0	8·5	2·9	2·9	5·7	35·0
6		53·0	7·8	5·8	10·0	23·3	77·5
7		56·3	25·2	1·5	3·7	13·3	135·0
8		12·6	66·4	1·7	14·9	4·6	87·5
9		18·7	66·6	1·3	8·0	5·3	37·5
10		72·0	15·7	5·6	4·5	2·2	44·5
11		23·3	0·0	17·6	17·6	41·2	17·0
12		23·6	54·5	3·5	16·0	2·3	1440·0
Totals		595·5	317·5	67·0	109·0	109·7	1971·0
Mean values		49·63	26·46	5·58	9·09	9·14	—
Percentage of total no. of larvae set		30·8	47·4	3·6	13·7	4·5	—
13	Transparent panels (Perspex) illuminated from above	35·8	22·8	4·1	9·8	27·6	61·5
14		26·9	9·5	5·6	15·6	42·5	115·5
Totals		62·7	32·3	9·7	25·4	70·1	177·0
Mean values		31·35	16·15	4·85	12·70	35·05	—
Percentage of total no. of larvae set		30·0	14·1	5·1	13·6	37·3	—
8	Opaque panels (Tufnol) illuminated from below	17·5	25·0	20·0	30·0	7·5	40·0
9		3·0	9·1	0·0	51·5	36·2	33·0
10		9·5	12·7	4·8	54·0	19·1	63·0
11		6·9	29·4	19·5	32·9	11·3	115·5
12		4·9	11·1	16·9	33·2	34·0	699·0
Totals		41·8	87·3	61·2	201·6	108·1	950·5
Mean values		8·36	17·46	12·24	40·32	21·62	—
Percentage of total no. of larvae set		5·6	14·0	16·0	35·1	29·2	—
13	Transparent panels (Perspex) illuminated from below	3·0	18·5	32·3	40·5	5·5	199·5
14		13·3	5·1	19·4	53·0	9·2	98·0
Totals		16·3	23·6	51·7	93·5	14·7	279·5
Mean values		8·15	11·80	25·85	46·75	7·35	—
Percentage of total no. of larvae set		6·4	14·1	28·1	44·7	6·7	—
2	Panels in total darkness (2–11, Tufnol)	50·0	0·0	0·0	0·0	50·0	2·0
3		22·2	22·2	11·1	28·5	15·9	31·5
4		0·0	0·0	0·0	66·7	33·3	3·0
5		0·0	0·0	100·0	0·0	0·0	1·0
6		27·7	18·1	15·7	20·5	18·1	83·0
7		35·0	23·4	8·0	19·0	14·6	68·5
8		21·0	25·3	3·2	21·0	29·5	47·5
9		26·5	38·2	2·9	23·6	8·8	34·0
10		18·2	33·3	12·1	18·2	18·2	33·0
11		34·3	8·6	31·4	5·7	20·0	35·0
13	(13–14, Perspex)	16·1	1·0	36·7	34·2	12·1	99·5
14		22·6	16·8	27·4	16·8	16·4	208·0
Totals		273·6	186·9	248·5	254·2	236·9	645·5
Mean values		22·80	15·58	20·71	21·08	19·74	—
Percentage of total no. of larvae set		24·0	17·5	20·7	21·1	16·8	—

being otherwise surrounded by a blacked-out box. In other experiments the tank was covered so that the experiment was conducted in the dark.

The results obtained using top light and opaque panels will be considered first (Fig. 4*b*). It is at once apparent that the trend of these results is very different from those discussed in the previous section. Here the 'normal' results have been obtained, with the highest settlement on the 0° surface; lower, but still high, on the 45° surface; and low on the others. It is not clear why the field results should have been so different. It is possible that the turbidity of the water in the Menai Straits causes a scattering of the light so that, even quite near the surface, the illumination is not markedly directional; this might be especially true in the dim light below Menai Bridge pier.

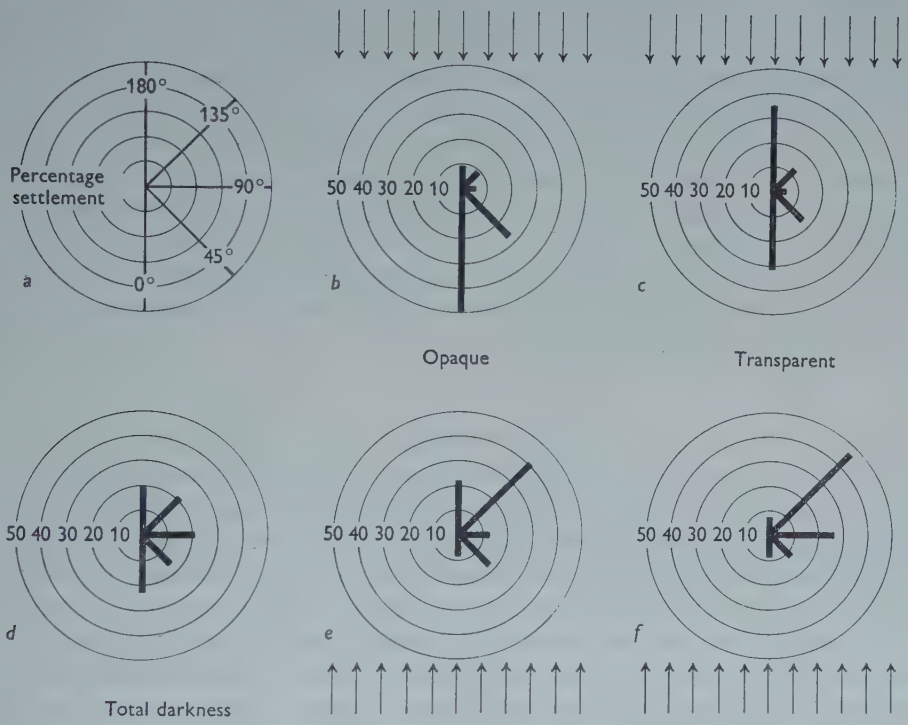

Fig. 4. The percentage settlement of *Celleporella hyalina* larvae on variously orientated panels. (*a*) Explanatory diagram (for further explanation see text). (*b*) Opaque panels illuminated from above. (*c*) Transparent panels illuminated from above. (*d*) Panels in darkness. (*e*) Opaque panels illuminated from below. (*f*) Transparent panels illuminated from below.

When the lighting of the tanks was reversed and the panels illuminated from below, the settlement pattern abruptly changed (Fig. 4*e*): the majority of larvae settled on the 180° and 135° surfaces—that is, on the shaded side of the panels.

In total darkness it can be seen that settlement on all five surfaces was approximately equal (Fig. 4*d*).

The results using top light and transparent panels were quite different. Settlement was highest on the horizontal panel, much lower on the oblique panel, and lowest on the vertical panel; but in each case it was more or less divided between the two surfaces (Fig. 4*c*). These results might be expected if the larvae tended to swim up and down rather than in a random manner—and this seems possible in view of the responses discussed earlier (p. 786). It is perhaps a little surprising that settlement on the two surfaces of the horizontal panel was about equal, for if the larvae were at first positive to light and then swam away from it, a heavier settlement might have been expected on the upper surface.

The results obtained when transparent panels were illuminated from below appear to be somewhat anomalous, the high settlement on the 135° surface being difficult to explain (Fig. 4*f*). This result, based on two experiments only, may have been due to uneven illumination, or perhaps to some unknown factor. In spite of this anomaly, however, the general picture to emerge from these experiments is clear. The pattern of settlement depends on the direction of illumination, and on opaque panels the larvae settle on the side facing away from the light.

Hyman (1959), reviewing previous work, drew the tentative conclusion that: 'Avoidance of light probably accounts for the selection of the undersurface of horizontal or nearly horizontal plates or other objects and the avoidance of surfaces much tilted from the horizontal.' The results just discussed show that, for *C. hyalina* at least, this can be regarded as certainly true. It seems quite likely that experiments with other marine larvae would show that the preference for 0° and, to a lesser extent, 45° opaque surfaces recorded by previous workers was also due to the selection of a shaded surface.

DISCUSSION

The behaviour shown by larvae in the troughs as a response to directional illumination appears to be a photoklinotaxis (Fraenkel & Gunn, 1940). The larvae swim along the light beam, either toward or away from the source. Their path follows a spiral course, for their ciliary action causes the larvae to rotate as they swim. Many of the larvae are provided with orange-red pigment spots; for example, those of *Bugula plumosa* have four and those of *B. flabellata* and *B. fulva* ten. In the literature dealing with the Polyzoa these have often been called eye-spots, but there is no evidence that they are photoreceptors (Hyman, 1959). Species like *Alcyonidium hirsutum* show equally distinct orientation to light yet lack pigment spots. The mechanism of photoreception remains unknown.

McDougall (1943) found that *Bugula neritina* larvae settled preferentially in the darkest of a series of variously illuminated chambers. He, and also Hyman (1959), regarded this preference for a low light intensity as evidence that the larvae become negatively phototactic (see p. 790). In fact, some sort of high photokinesis might better explain McDougall's finding of heavy settlement correlated with low light intensity. An experimental approach with this in mind might resolve the apparent paradox of the settlement behaviour of this species. It certainly seems important to show by suitable experiments whether or not polyzoan larvae can react to the intensity as well as to the direction of illumination.

The pattern of settlement on the variously orientated panels, which showed that the larvae metamorphosed on the shaded side, could also be the result of a high photokinesis causing the larvae to accumulate in the dark area. The present experiments have not resolved this point because *Celleporella hyalina* had already been shown to develop negative phototaxis; it would be of interest to repeat the experiments with a species which apparently displays positive phototaxis throughout its life.

SUMMARY

1. An apparatus is described for investigating the photic responses of marine larvae. A parallel beam of light is projected along horizontal Perspex troughs containing the larvae, while the use of a water bath prevents their being subjected to a rise in temperature.

2. Using a light beam of 5000 lux at normal summer sea temperatures, plotting the positions of the larvae in the troughs at regular intervals has revealed four types of behaviour:

I. Larvae at first all photopositive, later all becoming photonegative; for example, *Celleporella hyalina.*

II. Larvae at first all photopositive, only a small proportion of them later becoming photonegative; for example, *Bugula neritina.*

III. Larvae photopositive throughout the length of their natant phase; for example, *Flustrellidra hispida.*

IV. Larvae showing no response at all to directional illumination; for example, *Alcyonidium polyoum.*

3. It has been shown that the reversal of the sign of reaction to light in *Celleporella hyalina* larvae is dependent on the passage of time; it does not require their exposure to a certain amount of light for its inception.

4. When disks cut from *Fucus serratus* fronds were placed in the troughs as a substrate for settlement, the majority of *Celleporella hyalina* larvae developed a photonegative reaction before they metamorphosed.

5. Horizontally and vertically placed panels in the Menai Straits received settlement of polyzoans on all surfaces free from sediment and dense algal cover. These adverse factors would have masked any selection based on the behavioural responses of the larvae.

6. Laboratory experiments with opaque panels suspended vertically, obliquely and horizontally have shown that *Celleporella hyalina* larvae settle on the side away from the light. Thus they normally settle on the undersurfaces of horizontal and oblique panels, but this pattern is reversed if the panels are lit from below. Settlement on panels in the sea would therefore seem to depend on the reactions of larvae to light as well as on the unfavourability of upward-facing surfaces.

7. The nature of the larval responses is discussed.

I would like to express my gratitude to Dr D. J. Crisp for his interest in all stages of this work and for his helpful criticism of the manuscript, and to Prof. E. W.

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THE MODE OF OPERATION OF THE ELECTRIC RECEPTORS IN *GYMNARCHUS NILOTICUS*

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The perception of objects by *Gymnarchus niloticus* and similar electric fish has been attributed to an electrical mechanism (Lissmann, 1951, 1958). In a previous paper (Lissmann & Machin, 1958, hereafter referred to as L & M) we confirmed this hypothesis experimentally, and postulated a possible mode of action of the electric receptors. In the present paper experimental results are given which appear to confirm this postulate.

TWO POSSIBLE MODES OF ACTION OF ELECTRIC RECEPTORS

The electric receptors which, following L & M, will be assumed to be the mormyromasts, must measure the amplitude of a signal consisting of 1 msec. pulses at a repetition frequency of about 300 cyc./sec.; these pulses are emitted by the electric organ. The information about the signal amplitude has to be coded and transmitted down a sensory nerve in the form of impulses with a maximum rate of the order of 500 cyc./sec. It was proposed in L & M that this could only be done by 'smoothing' the incoming pulses and transmitting a sensory nerve signal characteristic of their mean value. An integration time constant of about $\frac{1}{4}$ sec. was suggested for the smoothing mechanism.

One other possible mechanism had been overlooked; this has been pointed out in a private communication by T. H. Bullock. According to Bullock's hypothesis, each signal pulse initiates a nerve impulse after a delay; this delay is dependent on the amplitude of the signal pulse. Since the signal pulse derives from the fish's own electric organ, its time of occurrence is presumably available in the C.N.S.; the amplitude of signal at the receptor can be deduced by comparing the timing of the transmitted pulses from the electric organ with that of the receptor nerve impulses. The reduction of band-width, or temporal integration, necessary to achieve adequate signal-to-noise ratio would then be carried out in the C.N.S. after the comparison has been made. This mechanism also gives an effective improvement of $\sqrt{3}$ in signal-to-noise ratio over the alternative mechanism (L & M, p. 476).

The mechanism postulated in L & M will hereafter be called 'pulse-frequency-modulation', while Bullock's mechanism will be known as 'pulse-phase-modulation'; both are illustrated in Fig. 1. The experiments described in this paper were designed to distinguish between the two mechanisms.

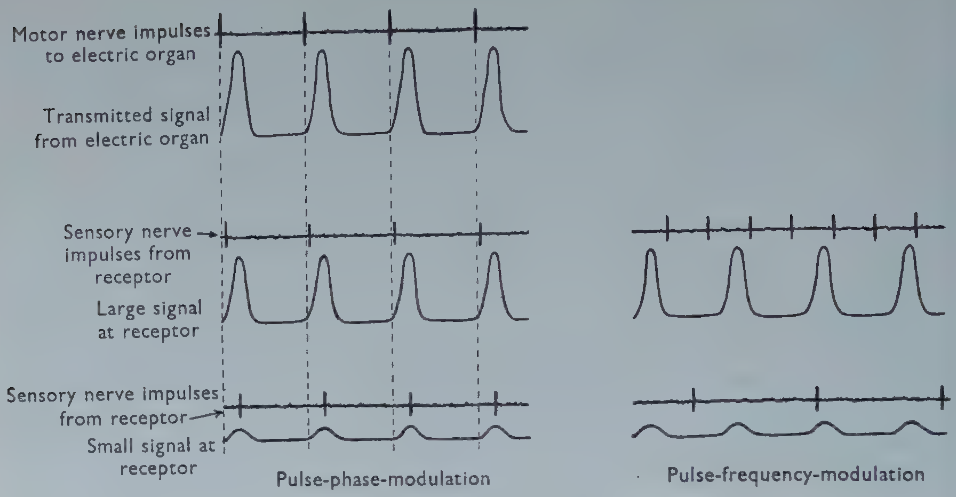
Motor nerve impulses to electric organ
Transmitted signal from electric organ
Sensory nerve impulses from receptor
Large signal at receptor
Sensory nerve impulses from receptor
Small signal at receptor
Pulse-phase-modulation
Pulse-frequency-modulation

Fig. 1. Comparison of two possible modes of action of the electric receptors.

PLAN OF EXPERIMENTS: RESULTS TO BE EXPECTED ON THE TWO HYPOTHESES

It was estimated previously (L & M) that *Gymnarchus* was sensitive to d.c. potential gradients of about 0·03 μV./cm. It was concluded (L & M, p. 477) that the same receptors were probably responsible both for object location and for the sensitivity to small direct currents. The present experiments are founded on the assumption that the sensitivity of the fish to externally applied currents gives information about the electric receptors used for object location.

If pulses of voltage are applied across the water in a tank, on the pulse-frequency-modulation hypothesis the fish should be affected only by their mean level. Thus square pulses of voltage V, width W and frequency f will have an effect dependent only on the mean level, given by VWf. *All* pulse trains with the same value of VWf should give the same effect provided that W and $1/f$ are not comparable with the smoothing time constant; furthermore the threshold sensitivity V_t for pulses of constant frequency should be inversely proportional to their width, and for pulses of constant width should be inversely proportional to their frequency. For applied pulses at the fish's own transmitted frequency, there is no reason to expect an enhanced sensitivity.

In the case of the pulse-phase-modulation mechanism, there is at first sight no possibility of registering the amplitude of applied pulses, since the moment of occurrence of the pulse is not available in the C.N.S. However, we may postulate that there are receptors which give an immediate response (i.e. not delayed) to an incoming pulse; the output from these receptors may provide the time reference from which the amplitude of the pulses can be deduced in the C.N.S. If the delay in firing of the nerve impulses is merely determined by the amplitude of the incoming pulse, its width should have no effect on the response.

THE MEASUREMENT OF INTEGRATION TIME-CONSTANT

It will be shown later that the experimental results favour the pulse-frequency-modulation mechanism. Assuming that this is the operative one, an approximate experimental determination of the integration time-constant τ can be made. The response of the fish will be dependent approximately on the mean value of the stimulus over the previous τ seconds. With a *single* pulse of voltage V' and width W' used as stimulus, the response should depend on $V'W'/\tau$. If now the threshold for continuous stimulation is V_t, and that for single pulse stimulation is $(V'W')_t$, then the integration time-constant is given by $\tau = (V'W')_t \div V_t$. If the threshold is found to be dependent only on $V'W'$ and not on V' or W' separately, further support is given to the pulse-frequency-modulation hypothesis.

MATERIALS AND METHODS

The experiments were carried out with a 50 cm. specimen of *Gymnarchus niloticus*, under ordinary laboratory conditions, in water at a temperature between 25 and 28° C. The experimental tank measured 120 × 75 × 45 cm. and contained two carbon plates 30 × 30 cm. mounted as shown in Fig. 2.

The electrical circuit providing pulses to the plates must have the following properties:

(i) It must be possible to switch the pulses on and off without changing the impedance of the circuit between the plates, so that any currents due to impurities in the plates (L & M, p. 453) are not altered at the instant of switching.

(ii) The frequency, amplitude and width of the pulses must be variable without changing the impedance in the plate circuit.

(iii) The plate circuit must be entirely insulated from earth, so that the effect of earth leakage from the water in the tank is minimized.

The circuit finally adopted is shown in Fig. 3. A standard electrophysiological stimulator provides pulses of variable frequency and width to modulate a radio-frequency signal generator. The signal generator has a piston attenuator, the output of which is fed to a transformer and rectifier circuit. Direct-current pulses isolated from earth and of widely variable amplitude are thereby generated. By switching the signal generator to the unmodulated condition, a d.c. voltage, variable by the attenuator, can be fed to the carbon plates.

The attenuator was calibrated in terms of voltage by amplifying the output of the rectifier and displaying it on a measuring oscillograph. Checks were carried out to ensure that the amplitude of the output pulses was independent of their frequency and width.

Before the experiments were started the fish normally either cruised about in the aquarium, or waited in one corner where it usually received its food. These activities were periodically interrupted by visits to the surface for a gulp of air.

When the carbon plates were first introduced into the aquarium the behaviour changed abruptly. The fish now rested most of the time on the bottom as far away

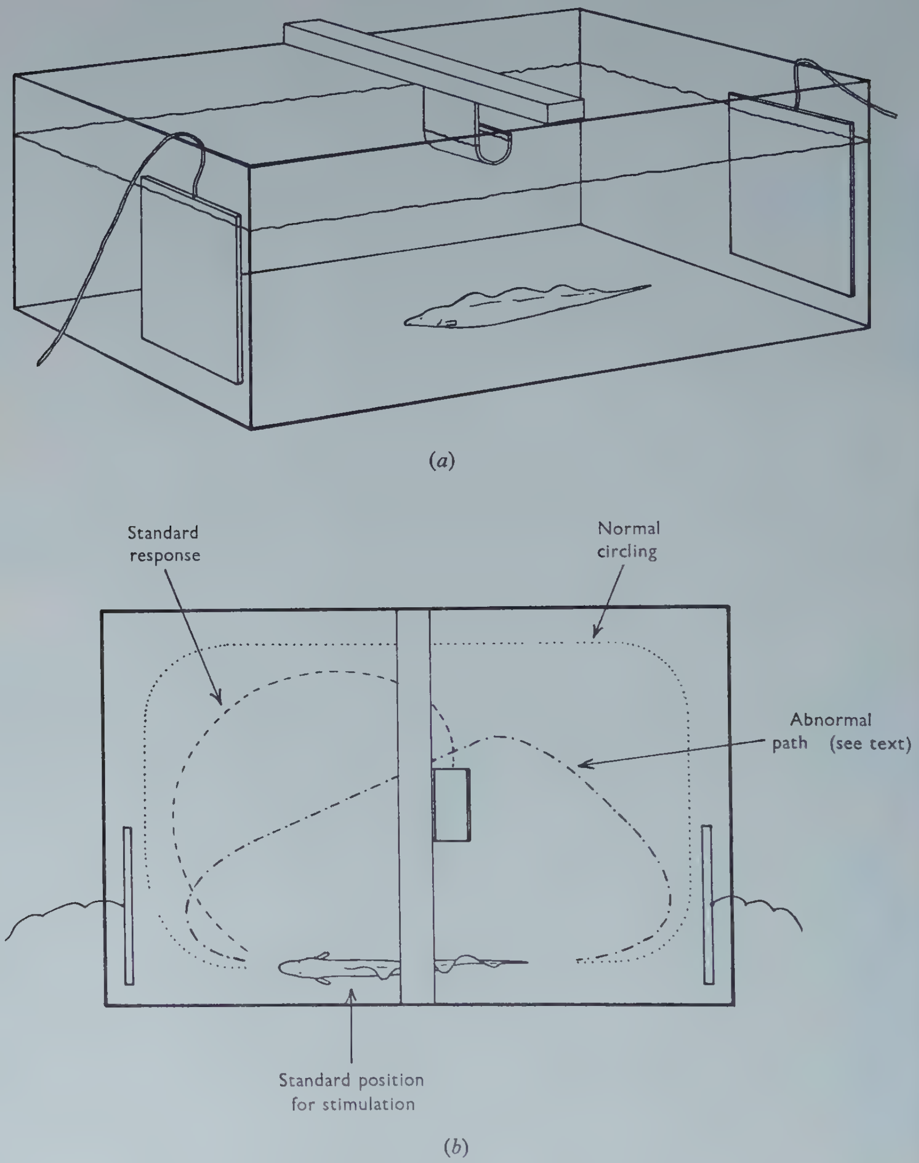

Fig. 2. Arrangements of the experimental tank. (*a*) General view. (*b*) Plan view.

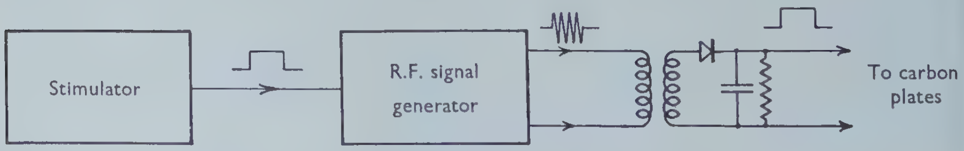

Fig. 3. Stimulating circuit.

from the carbon plates as possible. From time to time it undertook excursions towards the plates, swimming backwards and performing waving exploratory movements with the tail. After about 1 week it returned to its original pattern of behaviour and took no further notice of the plates.

When the fish was swimming between the carbon plates the application of an electric stimulus via these plates, if above a certain level, led to a sudden acceleration of the swimming movements. The stimulus consisted of either a single pulse or a pulse train about $\frac{1}{2}$ sec. long. In order to establish a more definite and standard response a series of training experiments was carried out. For this purpose a Perspex trough 10 cm. long and 5 cm. deep was placed close to the surface in the centre of the tank. After the application of the electrical stimulus, with the fish in a standard position between the plates, a piece of food suspended by a thread was presented. This reward was then used to lure the fish gradually nearer and nearer to the trough, and finally it was made to enter the trough before receiving the reward. From then on the fish was only fed when it had entered the feeding trough after the stimulus.

The normal course of an experiment was as follows: at first the fish was either stationary in one corner or cruising about. The introduction of the feeding trough led to steady circling along the walls of the tank. For reasons best known to itself the fish invariably circled clockwise on these occasions. Administration of an electrical stimulus above threshold brought about a marked increase in the swimming speed of the fish, a direct approach to the feeding trough and entry from the side furthest from the carbon plates. Thereupon the fish was fed. This is the 'standard response'. Application of a subthreshold stimulus caused no reaction and the fish continued to circle steadily.

The positive reactions were usually quite definite and left little doubt about the position of the threshold.

However, in the course of training several new patterns of behaviour emerged and some of these caused difficulties.

(1) Particularly during experimental sessions, the path of the fish along the wall of the aquarium furthest from the carbon plates was shortened by cutting off the corners, so that the fish finally swam on a more triangular course with the apex just beyond the trough (Fig. 2*b*).

(2) Between the carbon plates the fish often slowed down—as if waiting for the stimulus. Along this stretch of the path the tail was often waved markedly from side to side.

(3) Even in the absence of a preceding stimulus the fish at times hesitated or stopped near the entrance to the feeding trough.

(4) A disturbing feature appeared in the course of prolonged series of trials near the threshold level. The fish sometimes showed the very characteristic standard response (acceleration and entering the feeding trough) without any previous application of an electrical stimulus. Attempts to punish the fish on these occasions seemed to produce a neurotic state which took the form of aggressive behaviour, with the fish careering on the surface, its head out of water and snapping in all

directions. However, such apparently spurious positive responses appeared quite often to coincide with loud noises and vibrations in the building, for example, banging of distant doors or passing heavy traffic. Critical experiments were therefore undertaken at night, and generally the determination of the threshold was not only unambiguous but remained constant over a period of months.

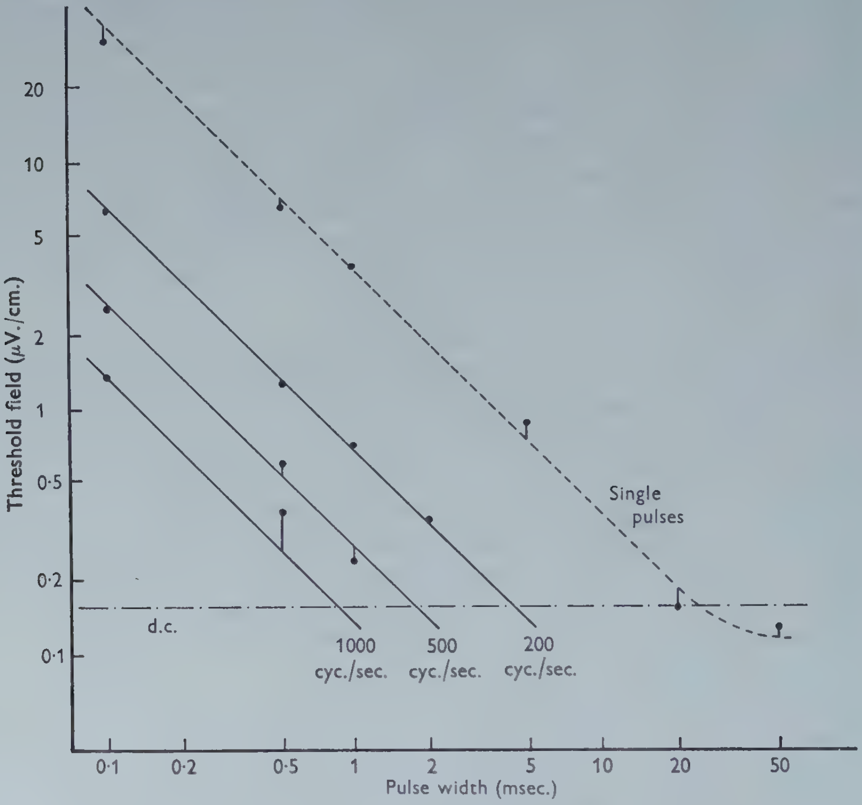

Fig. 4. Threshold field as a function of pulse width and frequency.

RESULTS

The threshold field for the standard response is plotted in Fig. 4 as a function of pulse width for three frequencies. The straight lines of slope −1 indicate the relation to be expected on the pulse-frequency-modulation hypothesis; their absolute position is chosen to give the best fit to all the results displayed. The horizontal line representing the threshold to d.c. agrees well with the pulse results, each sloping line cutting it at the appropriate point. Thus d.c. can be regarded as 1 msec. pulses at 1000 cyc./sec., or 5 msec. pulses at 200 cyc./sec., etc. It is clear that the results are consistent with the pulse-frequency-modulation mechanism.

The threshold for 1 msec. 200 cyc./sec. pulses was checked with the direction of field reversed. No significant change of threshold occurred.

The dotted line on Fig. 4 gives the threshold for single pulses; again the pulse-frequency-modulation hypothesis is confirmed. From the position of this line relative to those for repetitive stimulation, the integration time constant of the receptors is found to be 25 msec.

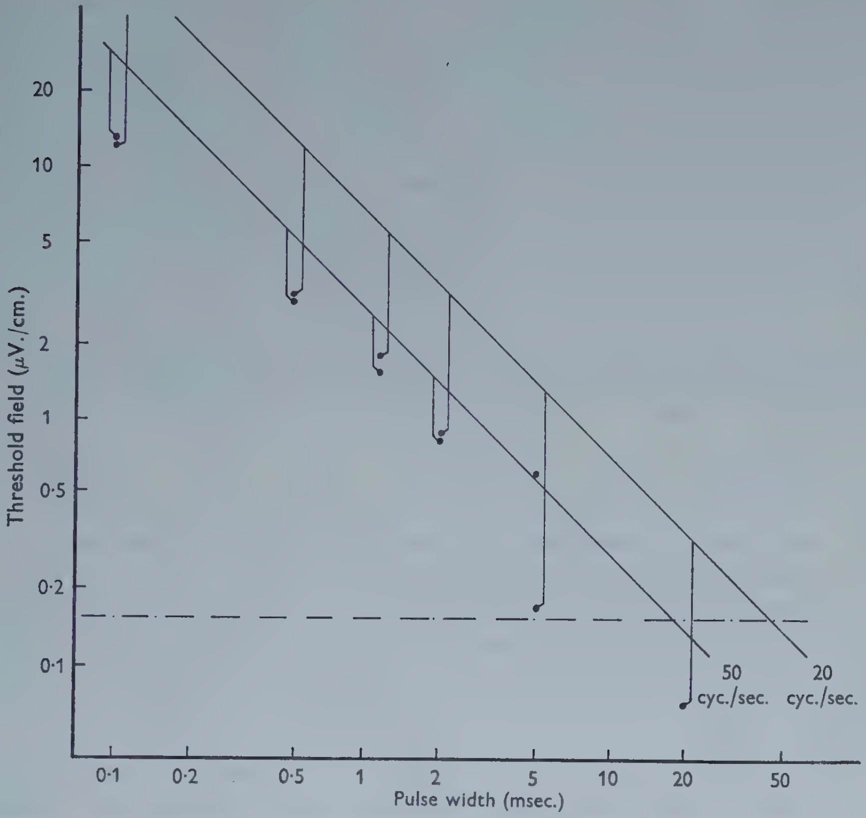

Fig. 5. Threshold field for 20 and 50 cyc./sec. pulses.

The results for a pulse repetition frequency of 50 and 20 cyc./sec. are shown separately in Fig. 5, together with the lines on which they should lie. When the pulse width or interval becomes comparable with the integration time constant it is apparent that the threshold is no longer inversely proportional to pulse frequency. Indeed the fish is more sensitive to 20 msec. pulses at 20 cyc./sec. than to d.c. Possible reasons for this will be discussed in the next section.

DISCUSSION OF RESULTS

Although the experimental results clearly favour our pulse-frequency-modulation hypothesis, it may be interesting to see whether the pulse-phase-modulation mechanism could give the same results. Each nerve impulse must now give information about the charge (current × time) passed during the pulse; therefore the impulse cannot be released until the whole of the stimulating pulse has occurred. The receptor must then comprise an integrating mechanism to measure current × time, an 'off' indicator to give the moment of termination of the pulse, a delay mechanism initiated by the 'off' indicator and of duration determined by the stored value of current × time, and finally a trigger for the nerve impulse. The time reference in the C.N.S. must be based on the 'off' indication. In this way a pulse-phase-modulation mechanism could be designed to give the observed experimental results, but only at the expense of extreme complexity of the receptors. No compensating advantages are obtained for the process of obstacle location, except possibly an improvement of $\sqrt{3}$ in the signal-to-noise ratio. It is still, however, impossible to explain the good fit of the d.c. stimulation results to those with pulse stimulation.

An explanation of the anomalous results at 50 and 20 cyc./sec. (Fig. 5) can be given on the pulse-frequency-modulation hypothesis by assuming that adaptation takes place before the transmission of information up the nerve to the C.N.S. When the stimulus is switched on the receptors integrate with a time constant of about 25 msec., then send a signal to the C.N.S. characteristic of the charge passed during that time. If the stimulus is continued the signal gradually ceases. Thus the sensitivity of the fish to single 20 and 50 msec. pulses is about the same as to d.c. (Fig. 4).

If, however, the stimulus is 20 msec. pulses at 20 cyc./sec., the receptors will send out a signal on the first pulse, 're-set' during the inter-pulse interval and send out a further signal for each subsequent pulse. Thus the effect at the C.N.S. of pulses spaced sufficiently far apart in time will be greater than that for a d.c. stimulus of the same voltage.

The adaptation and re-setting time is not likely to be less than the integration time-constant, and the results of Fig. 5 indicate that it is probably of the same order. Further experiments on this effect are being undertaken, and will be reported in a subsequent paper.

Adaptation at the receptors is clearly of advantage to the fish, as the process of obstacle location entails the detection of small changes in a standing current flowing in the receptors. The experiment described earlier, whereby the direction of the field was changed without changing the threshold, implies either that the receptors are sensitive to current of either polarity, or that two sets of receptors are provided, each sensitive to current of one polarity. When considered with the effect of adaptation, this suggests that changes in either direction of the standing current in the receptors could probably be detected.

It is possible that additional, less sensitive, receptors operating on the pulse-phase-modulation system are provided; the present experiments merely indicate that when the fish is operating near the threshold, this mechanism is not the operative one. It might, in fact, be advantageous to have some receptors which give information about the frequency of incoming pulses. 'Interference' with the locating mechanism due to stray currents in the water, or to the emissions of other nearby electric fish could then be identified. The problem of mutual interaction of electric fish in shoals, which could have been explained on a pulse-phase-modulation system, remains inexplicable on the present results. However, few field observations on the behaviour of social electric fish are available, and it is not clear whether or not mutual interaction confuses their locating mechanism.

The mechanism of temporal integration in the receptors

The smoothing of electrical stimuli by the receptors might be achieved by a simple resistance-capacity filter. The resistance could be identified with the jelly-filled canal leading to the mormyromasts, while one could postulate a relatively insulating membrane surrounding the mormyromast itself, giving it a capacity to the surrounding tissue. In L & M (p. 484) the resistance of a jelly-filled canal was estimated at 300 kΩ; assuming the mormyromast to be a sphere of 0·1 mm. diameter, and the membrane capacity to be 3 μF. cm.$^{-2}$ (Katz, 1952), the capacity would be 0·001 μF. The time-constant of such an integrating circuit would be about 0·3 msec.

It is conceivable that errors of a factor of 100 could have been made in the estimates of resistance and capacity, and that the smoothing time-constant is of structural origin, but this must remain speculative until accurate measurements on the jelly-filled canals and mormyromasts are possible.

The sensitivity of an electric receptor

Previous crude experiments (L & M, p. 451) suggested that *Gymnarchus niloticus* would respond to d.c. fields of about 0·03 μV./cm. The present results give a value of about 0·15 μV./cm., but are much more reliable. Previously there was a discrepancy of a factor of ten between the sensitivity derived from d.c. experiments and that inferred from object-location thresholds (L & M, p. 477). This discrepancy is reduced to a factor of two if the figure of 0·15 μV./cm. is used.

In L & M it was shown that the threshold sensitivity of *Gymnarchus* corresponds to a change of current in a receptor of 0·003 $\mu\mu$A. (This conclusion was based on the results of object-location experiments, and therefore corresponds to within a factor of two with the present results.) For a receptor integration time assumed there to be $\frac{1}{4}$ sec., this current will be only about 1/60 of the random noise current flowing in the receptor circuit. To explain this discrepancy it was assumed that spatial integration took place in the C.N.S. over about 5000 receptors, whereupon the signal-to-noise ratio would exceed unity. Averaging of the output of 5000 receptors would produce negligible spatial 'blurring' of the information.

The measured time-constant is one-tenth of the postulated value; it is therefore necessary to assume spatial integration over about 50,000 receptors. Mere averaging over such a large number would destroy some of the detail in the pattern of information from the receptor field. Accordingly, it must be assumed that the integration must take the form of a 'pattern recognition' process, whereby the pattern of information from the receptors is identified with one of a series of stored patterns, either innate or previously learned. Such a process is formally identical with spatial integration over a large number of receptors.

Even with spatial integration in the C.N.S., an individual receptor must transmit information about a current change of 0·003 $\mu\mu$A. This signal will be very 'noisy'; in terms of impulse transmission in the sensory nerve, it may represent an indiscernibly small change in the firing due to standing current and to noise. It is only when the outputs of 50,000 such information channels are correlated that a clear indication, free from noise, can be obtained.

The minimum detectable current is at first sight improbably small; it is of interest therefore to compare the sensitivity of electric receptors with that of other sense organs which respond to electrical stimuli. Such a comparison must inevitably be somewhat speculative. The present view about mechano-receptors involves the causal sequence stimulus → receptor potential → nerve impulses. It is tempting to identify the electric stimulus to an electric sense organ as the 'receptor potential', which initiates nerve impulses by a similar process. However, the values of receptor potential measured for mechano-receptors (Gray & Sato, 1953; Eyzaguirre & Kuffler, 1955) are of the order of millivolts at threshold; the corresponding currents are probably of the order of microamperes. It is, perhaps, not surprising that the currents involved in the operation of a mechano-receptor, which measures relatively gross mechanical changes, are much larger than those of electric receptors, which occur in very large numbers to provide essential orientation information for the fish. It would be more appropriate to compare electric receptors with the receptors of the ear or eye, where again essential orientation information is derived from the output of a very large number of receptors.

Tasaki, Davis & Legouix (1952) find in the ear of a guinea-pig cochlear microphonics which are linearly related to sound pressure. Extrapolating their results, the level of cochlear microphonic potential at the threshold of hearing should be about 0·01 μV. If the cochlear microphonics are the receptor potentials for the sense organs of the ear (although this is not universally accepted) and assuming that the resistance in the circuit to a sense organ is of the order of 1000 Ω, a current of about 10 $\mu\mu$A. should produce an output from the sense organ.

Direct evidence about the threshold of the eye to electric current is provided by Brindley (1955) who finds that a 50 cyc./sec. current of 20 μA. causes the sensation of light ('phosphene') of about 15° diameter. This corresponds to the excitation of about 3×10^6 rods and cones; the current per receptor is thus about 6 $\mu\mu$A.

The sensitivity attributed to the electric receptors of *Gymnarchus* is thus about a factor of 1000 better than the sense organs of the ear and eye. Since the electric receptors are presumably specialized for detecting electric currents while those of the ear and eye are not, such a factor is not unreasonable.

A current of 0·003 $\mu\mu$A. flowing for 25 msec. (the integration time of the electric receptor) corresponds to a movement of 1000 univalent ions. This figure is about a factor of ten lower than Brindley's (1955) value per rod and cone cell for the threshold for phosphenes produced by short electrical pulses. Such a threshold appears more than adequate when compared with a visual receptor, where the bleaching of one molecule of rhodopsin by one quantum can give rise to a signal (Brindley, 1960), or with an olfactory receptor, which can be stimulated by a single molecule (Neuhaus, 1953).

The mechanism by which such minute current changes can control the frequency of nerve impulses remains totally unexplained. However, even for the ear and eye, which have been studied for centuries, the situation is similar.

SUMMARY

1. Two possible modes of action of the electric receptors of *Gymnarchus niloticus* are described. In one, received pulses are 'smoothed' and a sensory discharge occurs dependent only on the smoothed level ('pulse-frequency-modulation'). According to the other mechanism, sensory nerve impulses are produced at the same frequency as the received pulses, but after a delay dependent on their amplitude ('pulse-phase-modulation').

2. An experiment has been devised to distinguish between the two mechanisms, and, if the pulse-frequency-modulation mechanism is the operative one, to measure the smoothing time-constant.

3. The results favour the pulse-frequency-modulation mechanism, and indicate a smoothing time-constant of 25 msec. It appears also that adaptation takes place in the electric receptors.

4. The origin of the smoothing action is discussed.

5. The sensitivity of electric receptors is compared with that of other sense organs.

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INHIBITION AND RHYTHMIC ACTIVITY OF THE CIRCULAR MUSCLES OF *CALLIACTIS PARASITICA* (COUCH)

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While much is known of excitatory phenomena in coelenterates, relatively little information is as yet available about inhibitory effects in these animals. Batham & Pantin (1954) have shown the existence, in *Metridium senile* (L.), of reciprocal inhibition between the parietal and circular muscles of the column and further postulate the presence of some inhibitory mechanism controlling the contractions of the circular muscles to permit peristaltic waves to pass down or up the column, although they also suggest that peristaltic wave formation may depend upon a slow conduction between muscle cells rather than on nervous activity. Among the Hydromedusae Horridge (1955) has shown that, following stimulation of its margin, the radial muscles of *Aequorea forskalea* P. & L. contract and that associated with this contraction is an inhibition of the activity of the circular muscle fibres responsible for the rhythmic contractions of the bell. A similar effect was demonstrated with representatives of many other genera of Hydromedusae.

Ross (1957) has shown the value of isolated rings of tissue in analysing the behaviour of the sphincter muscle of *Calliactis parasitica* (Couch), while Needler & Ross (1958) have demonstrated that the method is also applicable to the study of the activity of circular muscle from different levels of the column. The experiments described below have used this technique almost exclusively.

MATERIAL AND METHODS

Specimens of *Calliactis* were narcotized in a mixture of approximately equal volumes of sea water and isotonic magnesium chloride for at least 6 hr. before preparation. The column was then divided into a series of rings and mesenteries were trimmed down with fine scissors. These rings can be broadly characterized as subsphincter, mid-column, low mid-column and pedal rings. The ring including the sphincter muscle was discarded and a circular incision made through the base of the foot to cut away the greater part of the pedal disk. The division between low mid-column and pedal rings corresponds approximately to the level of the cinclides. After dissection the rings were allowed to recover for 36–48 hr. in sea water which was changed at intervals; during this period they lay passively and were not stretched.

The electrode assembly described by Ross (1957) was used to stimulate the rings with short condenser discharges from the type of apparatus devised by Hall &

Pantin (1937); the intensity of stimulation was generally about twice threshold. Recording was with lightly loaded gimbal levers, the preparation always being held at a slight tension. Latent periods were determined by stop watch. All experiments were conducted at room temperature which varied from 14 to 19° C. according to the time of year.

THE EFFECT OF CONTINUOUS STIMULATION

The effect of continuous low-frequency stimulation maintained for long periods depends upon the level from which the ring has been taken and also upon the age of the preparation. When a fresh subsphincter, mid-column or low mid-column ring is set up it will soon start to contract spontaneously, frequently in an almost regular rhythm. Each contraction lasts for about 3 or 4 min., the time interval between contractions varying from 7 to 25 min. Such a preparation, if left undisturbed, will continue to show spontaneous activity for long periods of time. If, after about 12 hr. of such spontaneous activity, the preparation is continuously stimulated at a frequency of one shock/2·75 sec.,* it will respond, after a latency of 30–40 sec., by the development of a strong contraction which is maintained, with minor alterations in length, for the duration of the stimulation (Fig. 1*a*). On cessation of stimulation tone is gradually lost during a period of apparent inactivity, spontaneous activity recommencing after an interval varying in different preparations from 5 to 20 min.

If, however, a fresh pedal ring is stimulated, a very different result is obtained (Fig. 1*b*). In this case either all activity ceases for the duration of the stimulation or only very slight activity is found. When stimulation ceases activity starts again after a period varying in different cases from 3 to 20 min. This type of behaviour is also typical of subsphincter, mid-column and low mid-column rings when they are first used. However, as these preparations age the inhibitory effect of stimulation becomes less marked; thus Fig. 1*c* and *d* show records of the response to continuous stimulation of the same low mid-column preparation, 90 min. and 12 hr. respectively, from the time it was originally set up. Pedal rings may continue to show the type of behaviour illustrated in Fig. 1*b* until they cease to be responsive. In some cases, however, an aged pedal ring preparation will show a different picture: initially there is a period varying from 10 to 30 min. during which no response to stimulation is recorded, but then a tetanic contraction follows which may or may not be held for the duration of the stimulation.

PEDAL RINGS

That electrical stimulation is excitatory to mid-column rings has already been reported by Needler & Ross (1958). It also has an excitatory effect on pedal rings. This is most easily seen by using a brief series of shocks. After a long but fairly constant latent period varying in different preparations from 2 to 5 min. there is

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* In what follows frequency of stimulation is expressed as the 'shock interval', that is the time interval between two successive shocks.

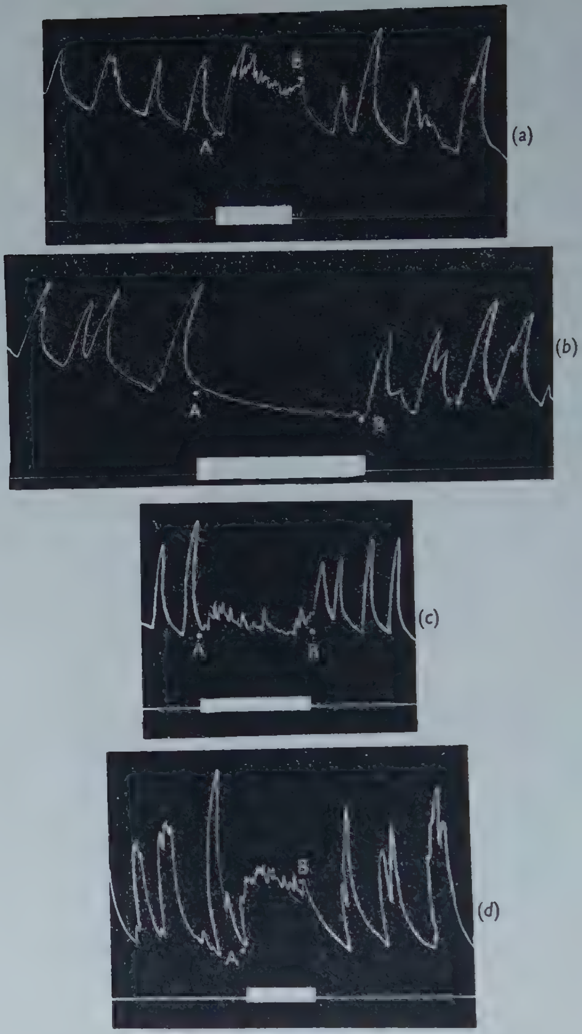


Fig. 1. *Calliactis parasitica.* Responses of circular muscle to continuous stimulation at a shock interval of 2·75 sec. In this and all subsequent records contractions are shown as upward movements of the trace. Stimulation starts in each record at the spot adjacent to the letter A and ceases at the spot adjacent to letter B. (*a*) Mid-column preparation. Duration of stimulation 17 min. (*b*) Pedal ring of the same specimen. Duration of stimulation 38 min. (*c*) Low mid-column ring. Stimulation started 90 min. after preparation first set up. Duration of stimulation 27 min. (*d*) Same preparation as (*c*) 12 hr. after first set up. Duration of stimulation 17 min.

a strong slow contraction which takes from 90 sec. to 3 min. to reach maximal shortening. Relaxation is equally slow. That these slow contractions, despite their long latency, are related to the stimulation and are not simply 'spontaneous' contractions which occurred subsequently is demonstrated in Fig. 2 where a series of ten shocks at a shock-interval of 2·75 sec. was applied at varying times from the onset of the previous contraction. It can be seen from Table 1 that the latencies of

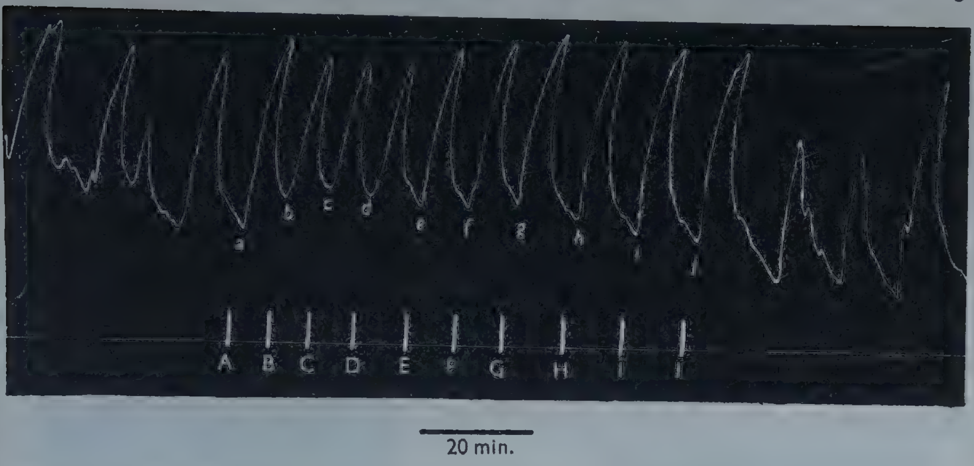


Fig. 2. *Calliactis parasitica.* Pedal ring. Responses to series of stimuli of ten shocks at a shock interval of 2·75 sec. at varying times from the preceding stimulus. Capital letters indicate each stimulus, small letters the corresponding contraction. See also Table 1.

Table 1. *Latencies of response of the circular muscle of a pedal ring of* Calliactis *stimulated at varying intervals from the preceding contraction. The data refer to the responses shown in Fig.* 2

Stimulus	Time from start of previous response (min.)	Latency (sec.)
B	4	120
C	4	173
D	4	140
E	6	119
F	6	110
G	6	128
H	8	118
I	8	115
J	8	100

these responses are not markedly affected by the interval between stimuli, while inspection of the record shows clearly that the contractions relate to the pattern of stimulation, the natural rhythm of activity of the preparation being far slower.

That brief stimulation of the pedal ring results also in an inhibition may be demonstrated in the following manner. A series of identical stimuli is given in regularly spaced intervals, timed from the onset of the previous response. If now, just as one of these contractions starts to develop, the preparation is again stimulated, the full development of the contraction is inhibited (Fig. 3).

If the number of shocks given is increased the latent period is increased (Table 2) while the magnitude of the contraction following is decreased. Prolonged series of shocks may be followed by only tiny contractions (Fig. 4).

While an increasing number of shocks invariably causes an increase in the value of the latency, the extent of this effect is variable. As can be seen from Table 2 its magnitude appears to be correlated with the age of the preparation. The readings

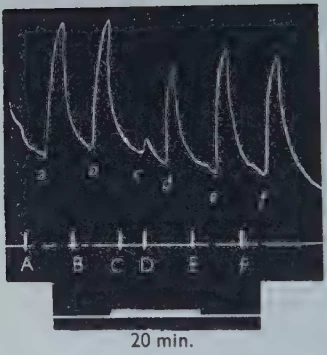


Fig. 3

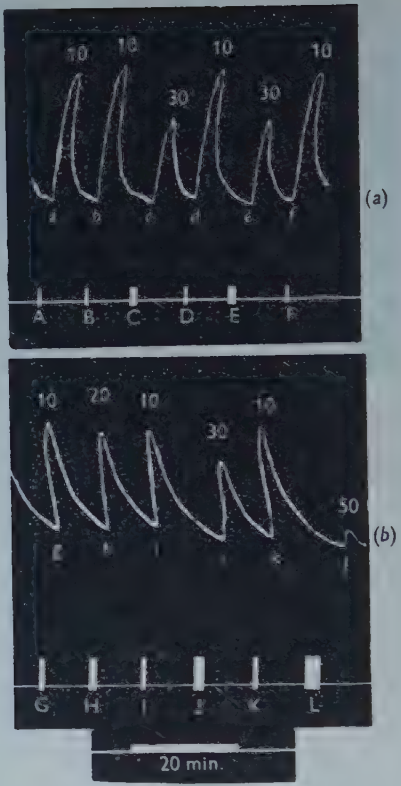


Fig. 4

Fig. 3. *Calliactis parasitica*. Pedal ring. Demonstration of the inhibitory effect of a short burst of shocks. Lettering convention as in Fig. 2. All stimulation ten shocks at a shock interval of 2·75 sec. Stimulation at B, C, E and F was 6 min. after the onset of the previous contraction. Stimulation at D was after 30 sec. Note that the stimulus D inhibits the development of the response C.

Fig. 4. *Calliactis parasitica*. Pedal rings. Effect of number of shocks on magnitude of response. All stimulation at a shock interval of 2·75 sec. and in each case after an interval of 6 min. from the onset of the previous contraction.

Table 2. *Effect of number of shocks upon the latency of response* (L, *in sec.*) *of the circular muscle of the pedal ring of* Calliactis parasitica

Preparation ...	AZ		BF		BH	
Age of preparation ...	3 days		4 days		4 days	
No. of shocks	L	N	L	N	L	N
10	173	6	172	7	138	8
15	226	6	—	—	—	—
20	293	4	208	3	153	3
30	425	1	263	3	159	3
50	—	—	344	1	186	1

N is the number of observations

with AZ were taken on the third day of use; similar results had been obtained from this preparation 2 days earlier, while 2 days later the results were similar to that of the preparation BF, itself in the fourth day of use. Preparation BH was also 4 days old and completely unresponsive the following day.

The increase in the value of the latent period with the number of shocks may be regarded as being due, in some manner, to an enhancement of inhibition. Making this assumption, it is possible to use an increase in the value of the latency to determine the minimal number of shocks which will produce an inhibitory effect. A series of shocks, say ten, is given and then, 2 min. later and before the onset of a response, another series. The effect upon the latent period of varying the number of shocks in the second series can be observed. Results from experiments such as these are summarized in Table 3. It should be emphasized that in any of these preparations, although the value of the latent period to a constant stimulus is reasonably consistent over a short period, it may change fairly abruptly. As a result in many experiments concerned with latent period a standard stimulus of ten shocks was used at regular intervals as a check upon such variation; further it was not usually possible to obtain large numbers of readings from a single preparation. An examination of Table 3 shows that three intercalated shocks markedly lengthen the latency, as do two. A single shock also, on the average, lengthens the latency but in this case there is considerable overlap with the control values; the difference is just significant at the 0·05 level in the first experiment, but not in the second. However, the number of observations is too small to allow any value to be attached to this statistical result.

Table 3. *Effect of intercalated shocks upon the latency of response* (L) *of the circular muscle of the pedal ring of* Calliactis parasitica

A standard stimulus of ten shocks at a shock interval of 2·75 sec. was given and then 120 sec. later one or more additional shocks at the same frequency were intercalated.

	Expt. 115			Expt. 130		
No. of intercalated shocks	L (sec.)	Range	N	L (sec.)	Range	N
0	168	142–184	11	146	104–192	17
1	184	168–206	5	179	135–230	4
2	225	217–230	3	222	187–235	5
3	242	239–244	2	—	—	—

N is the number of observations.

The problem may be approached in another way which is best described by taking a specific example. A pedal ring showing frequent steady rhythmical contractions is selected; in the immediate case these contractions occurred every 7 min. Five minutes after the start of such a contraction a single shock was applied to the preparation; this resulted in a strong contraction and there followed a pause of 7 min. before another spontaneous contraction occurred. The preparation was allowed to make two further spontaneous contractions and 5 min. later two shocks, separated by an interval of 2 min. 33 sec., were given. A further spontaneous contraction did not occur until after an interval of 13 min. Again after two further spontaneous contractions three shocks at the same interval as before were applied and the pause before the next spontaneous contraction lengthened to 25 min. It is clear from this type of result that single shocks can be inhibitory in effect.

Table 4. *Effect of frequency of stimulation upon the latency of response* (L) *of the circular muscle of the pedal ring of* Calliactis parasitica

Shock interval (sec.)	Expt. 117			Expt. 135			Expt. 136			Expt. 147		
	L (sec.)	Range	N	L (sec.)	Range	N	L (sec.)	Range	N	L (sec.)	Range	N
1·1	161	150–178	3	191	180–200	3	118	98–132	5	—	—	—
2·75	154	148–164	4	182	172–193	6	105	96–117	5	142	134–156	3
6·80	171	167–175	3	163	152–178	3	—	—		132	123–143	3

N is the number of observations.

The effect of stimulation frequency upon latent period has also been examined. The results of a series of such experiments are shown in Table 4. As with other determinations of latency, the number of observations which can be made on any one preparation is small, but the figures suggest that there is no effect of stimulation frequency upon latency, or if any effect does exist it is very small compared with the influence of the number of shocks.

The inhibitory effect of stimulation may finally be demonstrated by its action upon the rate of relaxation from a slow contraction. If a preparation is stimulated by a short battery of shocks and then, as maximal shortening is reached, the preparation is again stimulated, the rate of relaxation is far more rapid than in a preparation which is not stimulated a second time. Such an effect is shown in Fig. 5*a* and *b*. While any accurate assessment is impossible, an estimate of latency of the inhibitory effect can be obtained from these experiments. It is relatively brief, of the order of 15–20 sec.

In sum, the foregoing experiments demonstrate that electrical shocks applied to pedal rings of *Calliactis* have a dual effect, stimulating both an excitatory and an inhibitory system which control the activity of the circular muscles; and further that such inhibition can also exist in more adoral regions of the column though the extent of its expression varies with the age of the preparation. A more detailed discussion of the results follows later.

TRANSFORMATION OF THE RESPONSE IN THE MID-COLUMN

If the latency of a pedal ring is compared with that of a mid-column ring which has been set up and active for many hours, they are seen to be markedly different. Such mid-column preparations show latencies of 30–40 sec., while values of the order of 120 sec. or more are typical of pedal rings. The latency of response of fresh mid-column preparations is, however, also of the order of 120 sec., but very soon a small initial contraction appears with a far shorter latency and, as time passes, this contraction becomes dominant. Fig. 6 shows a series of contractions from the same preparation taken at hourly intervals, while Table 5 records the latencies of these responses. It will be seen that initially there is a clear-cut double response, but that with the growth of the first contraction the two gradually merge. The latency of the onset of the second contraction does not, however, significantly alter. This same pattern of transformation has been observed in a considerable number of preparations.

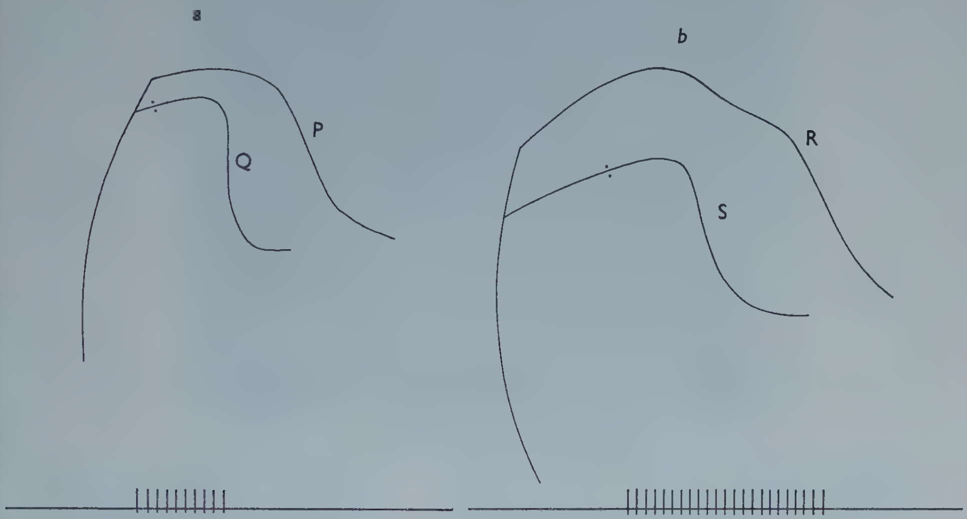


Fig. 5. *Calliactis parasitica.* Pedal rings. The effect of stimulation at maximal shortening. In *a* and *b* two consecutive recordings of responses to 10 shocks have been superimposed. The records shown are tracings from photographic negatives. In each case the drum was started 90 sec. after the onset of the contraction. In Q ten shocks were given 5 sec. later: in S twenty-two shocks were given 30 sec. later. The onset of stimulation is indicated by spots on the record. It will be seen that the rates of relaxation of Q and S are more rapid than those of P and R. All stimulation at a shock interval of 2·75 sec.

A number of pedal ring preparations show double responses similar to the contraction recorded after 2 hr. in Fig. 6. The latency of the initial response in these cases is about 50 sec. but unlike a mid-column preparation a pedal ring preparation shows almost no variation in the pattern of response with time.

RHYTHMICITY

As has already been stated, rings of tissue from regions below the sphincter show spontaneous activity which is often of a fairly regular and rhythmical character. The experiments now to be described attempt some analysis of the genesis of this rhythm.

The regular rhythmic contractions of these circular muscles recall, albeit on a far slower time scale, the beat of a medusa bell. The rhythmic beat of a medusa such as *Aurelia* has been shown to depend upon the presence of pace-makers in the ganglion masses of the tentaculocysts, although the muscles may also show rhythmic

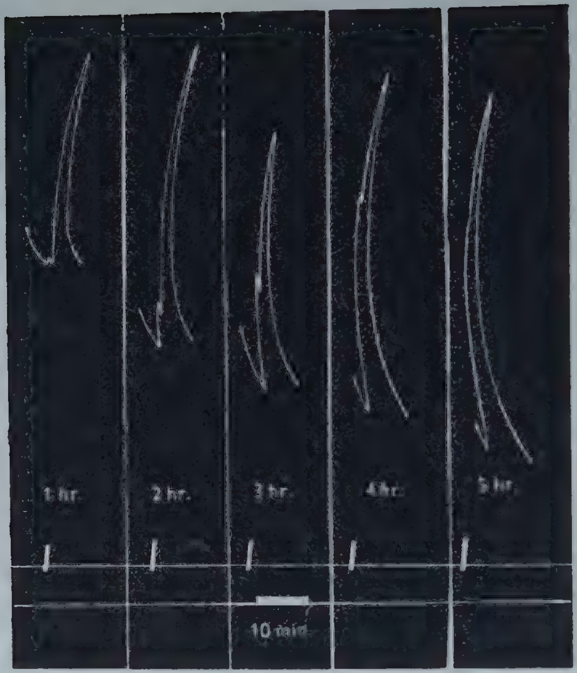
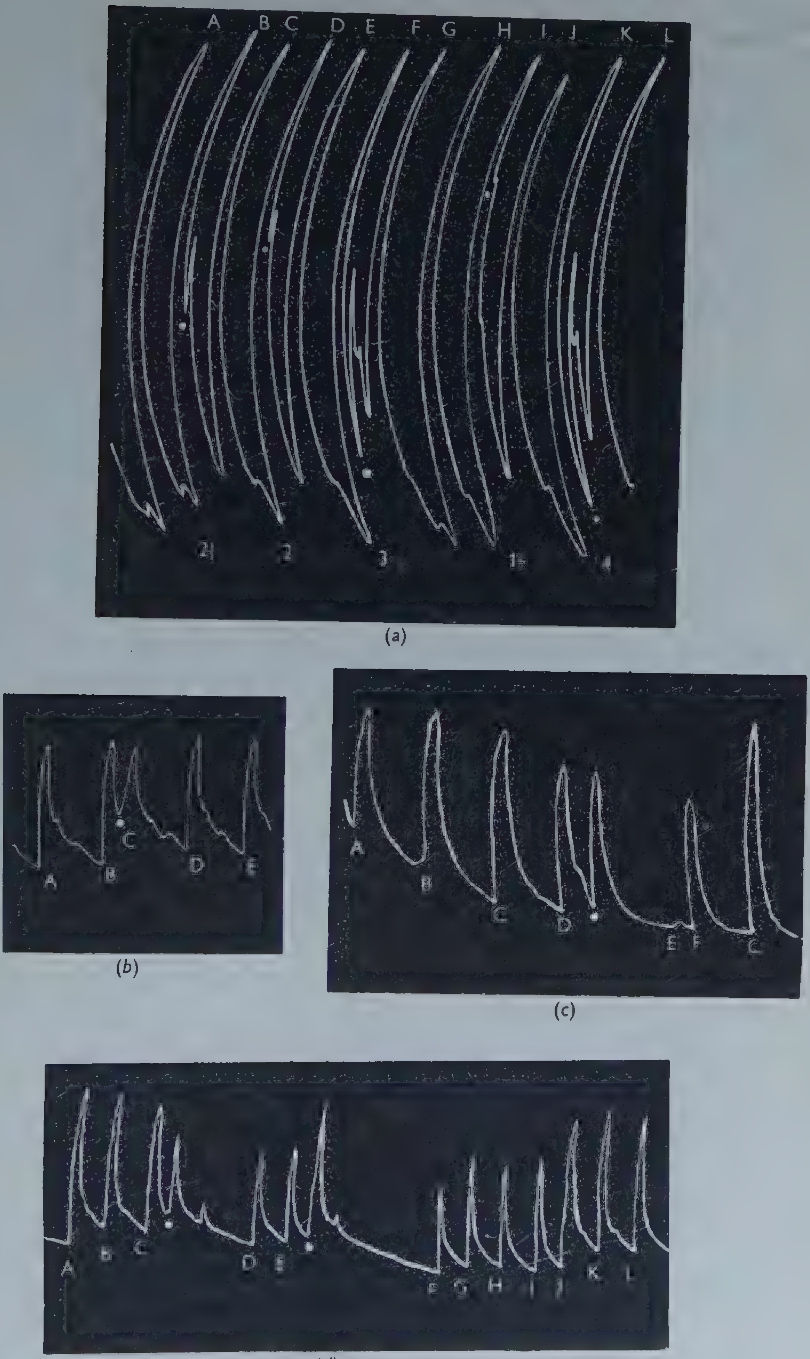


Fig. 6. *Calliactis parasitica.* Mid-column ring showing the change in character of the response with time from first setting up a preparation. In each case a standard stimulus of ten shocks at a shock interval of 2·75 sec. was given and then 6 min. after the start of the response, the same stimulus was applied. The records show only the response to the second stimulation in each case. Details of the latencies are shown in Table 5.

Table 5. *Latencies of the responses shown in Fig.* 6

Time from setting up (hr.)	Latency (sec.) to	
	First response	Second response
1	61	114
2	48	108
3	37	119
4	40	90
5	30	95

spontaneous activity when the tentaculocysts have been cut away (Pantin & Vianna Dias, 1952). Clearly there is no anatomically defined pace-maker in the circular muscle of *Calliactis*, but an attempt may be made to answer the question as to whether or not pace-maker function lies within whatever system is responding to electrical stimulation. As Pantin & Vianna Dias (1952) have elaborated, if the pace-maker is an integral part of the excited system, an intercalated stimulus will either reset the rhythmic activity or possibly result in a missed beat so that there follows a 'compensatory pause'. If, however, the pace-maker is not integral with the excited system an intercalated shock may have no effect or be followed by a compensatory pause.



(*a*)

(*b*)

(*c*)

(*d*)

Fig. 7. *Calliactis parasitica.* Effect of intercalated stimuli upon the rhythmic activity of the circular muscles of the mid-column. The response to each intercalated stimulus is indicated by a white dot. In (*a*) stimulation was at varying time intervals in min., as indicated on the trace, after the start of a spontaneous contraction. For further details see Table 6.

Table 6. *Numerical data relating to the experiments shown in Fig.* 7

Fig. 7*a*. Stimulation 20 shocks at a shock interval of 1·25 sec.

Time interval between contractions (min.)	A–B	B–C	C–D	D–E	E–F	F–G	G–H	H–I	I–J	J–K	K–L
	8	7	8	7	9	7	10	9	7	9	8

Fig. 7*b*. Stimulation by five shocks at a shock interval of 3·6 sec. 3 min. from the start of a spontaneous contraction.

Time interval between contractions (min.)	A–B	C–D	D–E
	13	13	11

Fig. 7*c*. Stimulation by twenty shocks at a shock interval of 1·25 sec. 6 min. from the start of spontaneous contraction.

Time interval between contractions (min.)	A–B	B–C	C–D	D–F	F–G
	12	12	13	24	11

Fig. 7*d*. Stimulation at a shock interval of 3·6 sec. 3 min. from the start of a spontaneous contraction. First stimulus twenty shocks: second stimulus five shocks.

Time interval between contractions (min.)	A–B	B–C	C–D	D–E	E–F	F–G	G–H	H–I	I–J	J–K	K–L
	7	8	21	7	30	6	7	7	6	7	7

Experiments of this type have been made upon a considerable number of mid-column preparations and a wide variety of effects encountered. Examples are shown in Fig. 7, details of the experiments illustrated being given in Table 6. Intercalated stimuli may have no effect (Fig. 7*a*); they may cause the rhythm to reset from the time of the intercalated contraction (Fig. 7*b*); an effect which could be interpreted as a compensatory pause is frequently found (Fig. 7*c*), while pauses longer than compensatory pauses may also occur (Fig. 7*d*). All four of these events appear to occur with about equal frequency. In twenty-four preparations five showed no alteration in rhythm, five showed a simple reset, ten showed what could be interpreted as a compensatory pause and four showed longer delays. It seems likely that, as in *Aurelia*, many of the cases which have been classified as compensatory pauses are not so in any simple way. Thus in Fig. 7*c* a small contraction E developed after the intercalated stimulus at a time corresponding to a simple reset of the rhythm. In Fig. 7*d* the two long pauses can be regarded as due to two and three missed beats, respectively, which would have taken their rhythm from the intercalated contractions; that is, in both cases the rhythm has been reset by the intercalated stimulus.

Despite numerous trials it has not been possible to demonstrate any consistent effect of the number or frequency of the shocks in an intercalated stimulus or of the time of application of the stimulus in relation to the normal rhythm.

It is clear that, even if the cases of compensatory pauses are disregarded as spurious, cases occur both of a reset of the rhythm and also of the stimulation not altering the rhythmic pattern of the contractions. This combination of events is not expected in terms of either hypothesis concerning the possible site of the pace-maker. It seems probable that the reason for this lies in the mistaken initial assumption that the effect of stimulation is purely excitatory to these preparations of *Calliactis* muscle. As we have seen in the preceding sections, electrical stimulation has both an excitatory and an inhibitory effect; the precise balance of these varies in mid-column preparations with time. As a result, if the stimulus is strongly excitatory it may have no effect upon the rhythm, while if it is at least partly inhibitory in action a greater or less delay may follow depending upon the exact balance of excitation and inhibition produced by the intercalated stimulation. If this interpretation is correct, it follows that when the stimulus is purely excitatory the effect of an intercalated stimulus is without effect on the rhythm, and this carries the implication that the pace-maker mechanism is not an integral part of the excited system. Such an interpretation is in keeping with a further analysis of the origin of the rhythmicity arising from a more detailed study of the behaviour of mid-column rings.

MID-COLUMN RINGS

So far detailed consideration of inhibitory phenomena has been limited to the behaviour of pedal rings and the question arises as to whether inhibitory effects may be demonstrated in mid-column preparations which have been active for many hours and show only a single response of short latency to electrical stimulation. We have seen above that when stimuli are applied in a regular programme to a pedal ring the magnitude of the shortening decreases and the latency increases with an increase in the number of shocks. This is partly true of mid-column preparations. Fig. 8*a* shows the responses of such a ring to a series of ten and to a series of thirty shocks. It will be seen that while the response is less for the greater number of shocks, the latency is unaffected by the number of shocks (Table 7). It can further be seen that following stimulation with thirty shocks the next response to a further thirty shocks is less and that the subsequent responses to groups of ten shocks gradually grow. As with the pedal rings the inhibitory effect of stimulation is clearly of long duration and it may be taken that the magnitude of a response is a reflexion of the balance between excitation and inhibition—an assumption which cannot be made with the pedal rings owing to the varying duration of the latency.

Granted this assumption, it is possible to use a test battery of stimuli to investigate the level of inhibition in a preparation following a standard stimulus. Such an experiment is shown in Fig. 8*b* where, following standard stimuli of ten shocks at a shock interval of 2·75 sec. test batteries of five shocks were applied at varying intervals after the onset of the contraction in response to the standard stimulus. It will be seen that as this interval increases the response to the test stimulus also increases in magnitude. The recorded latencies (Table 7) of the responses to the test stimuli are in general longer than those to the standard stimulus. This is almost certainly due to the fact that the former were measured to the time of first upward movement of the recording lever, while the true latency would be the time to the checking of the lever's downward movement during the relaxation from the standard stimulus. Lastly, it should be noted that the record ends with a final response to the test stimulus alone and that this is greater than the penultimate response which followed a standard stimulus by 4½ min. It is thus clear that even after this period the response to the test stimulus is still partially inhibited.

Fig. 8*c* shows a continuation of this type of experiment on the same preparation 2 hr. later with still longer intervals between standard and test stimulus. Details

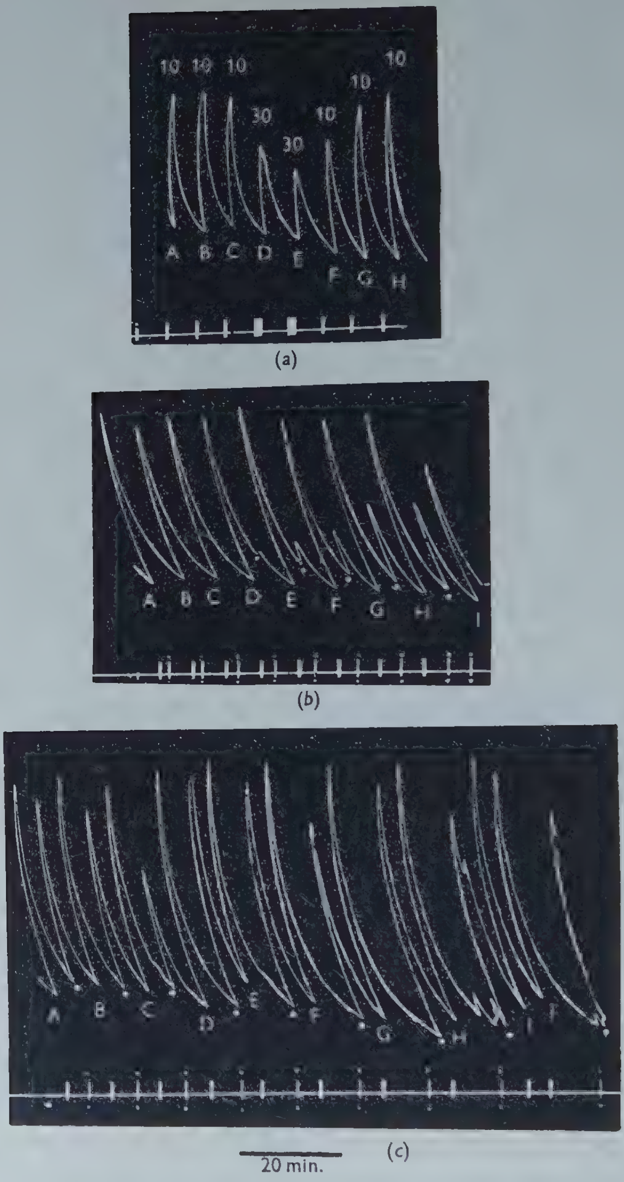


Fig. 8. *Calliactis parasitica*. Responses of the circular muscles of the mid column to different programmes of stimulation. Details of the programmes and latencies of the responses are shown in Table 7.

of stimuli and latencies are shown in Table 7. By this time the response to the test stimulus of five shocks 5 min. after the standard stimulus B was no greater than that 4 min. after the standard stimulus A. However, the response to a test stimulus of four shocks 5 min. after the standard stimulus C was markedly less. When the interval from the standard stimulus D to a test stimulus of four shocks was increased

Table 7. *Details of stimulation programme and latencies of responses of experiments illustrated in Fig.* 8

Fig. 8*a*. Responses to groups ten or thirty shocks at a shock interval of 2·75 sec. With the groups of ten shocks the preparation was stimulated 5 min. after the onset of the previous contraction: with the groups of thirty shocks the interval was taken as 5 min. from the cessation of the previous stimulation.

Response	A	B	C	D	E	F	G	H
Latency (sec.)	38	35	35	36	35	37	36	37

Fig. 8*b*. Responses to a standard stimulus of ten shocks at a shock interval of 2·75 sec. followed by a test stimulus of five shocks of the same frequency at varying intervals after the standard stimulus. The interval between the response to the test and the next standard stimulus is 4 min. In this and Fig. 8*c* both the test stimulus and the corresponding response are indicated by white dots.

Stimulus	No. of shocks	Latency (sec.)	Time to test stim. (min.)	Latency of test response (sec.)
A	10	33	1½	No response
B	10	35	1½	No response
C	10	36	2	No response
D	10	34	2½	56
E	10	35	3	43
F	10	37	3½	45
G	10	37	4	40
H	10	37	4½	40
I	5	37	—	—

Fig. 8*c*. As (*b*) but with varying numbers of test shocks. Interval between response to test stimulus and standard stimulus 4 min. except before D when it was 5 min. Interval between I and J was 4 min.

Stimulus	No. of shocks	Latency (sec.)	Time to test stim. (min.)	No. of test shocks	Latency to test stim. (sec.)
A	10	38	4	5	35
B	10	40	5	5	36
C	10	40	5	4	40
D	10	37	6	4	37
E	10	37	7	3	36
F	10	37	8	2	38
G	10	35	9	2	33
H	10	39	10	1	38
I	10	32	—	—	—
J	10	40	10	1	39

to 6 min., the response was greater and of the same magnitude as that shown to a test stimulus of three shocks 7 min. after the standard stimulus E. The response to two shocks 9 min. after the standard stimulus G is greater than that 8 min. after the standard stimulus F. Finally, the response to a single shock 10 min. after the standard stimulus H is almost maximal. That this latter response relates to the stimulating shock and is not simply a spontaneous contraction is shown by the fact that its latency is similar to that for responses to larger numbers of shocks. Fig. 9 shows high-speed recordings of responses from a different preparation to groups of shocks of varying number; it will be seen that the characteristics of the response are almost unaffected by the number of shocks applied.

It becomes clear from these results that the responses of a mid-column preparation are not absolute but will depend upon the previous stimulation applied to a preparation. As a result it is not possible to make a useful analysis of responses to different frequencies and numbers of shocks. What is, however, of prime importance in an understanding of the genesis of the rhythmic activity of these preparations is the demonstration of a slow variation in excitability.

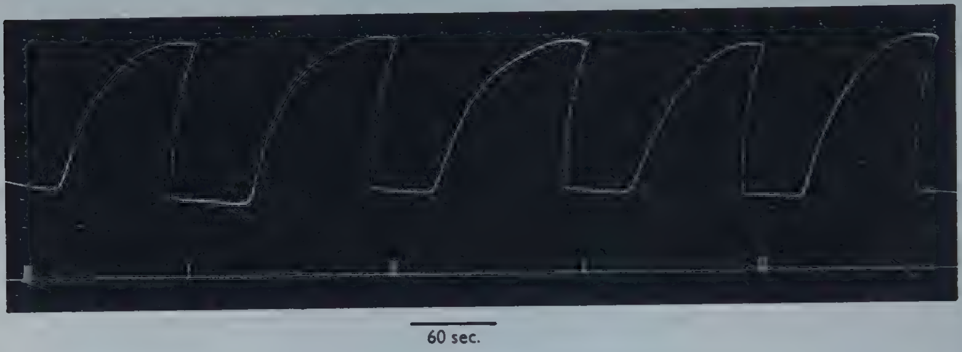


Fig. 9. *Calliactis parasitica.* Mid-column preparation. High-speed recordings of responses to different shock numbers. Kymograph stopped after contraction reached maximal extent in each case. Time interval between stimulations 7 min.

DISCUSSION

The preceding experiments have been concerned with two different phenomena in the circular muscles of the column and pedal ring of *Calliactis*, namely, inhibition and rhythmicity of spontaneous activity. These must be considered separately although, as will be suggested later, they may in fact be interrelated.

It is clear that electrical stimulation is not usually simply excitatory but has a dual action whose expression may change with the activity of the tissue. In a pedal ring we may visualize the long latencies recorded as being due to this dual action. On stimulation inhibitory effects dominate and then gradually die away until the balance of excitation to inhibition is reversed and a contraction follows. The duration of latent period thus becomes a measure of the relative magnitude of the inhibitory effect of stimulation. Evidence has been produced which shows that a single stimulus to a pedal ring can have an inhibitory action, and that the latency of the response is affected by the number of shocks applied (Table 2) but not by their frequency (Table 4). These facts suggest that each stimulus produces a quantum of inhibitory effect and that where many stimuli are given the latency is prolonged by the accumulation and subsequent dissipation of this inhibition.

Such an interpretation implies that the excitatory effects of stimulation persist for prolonged periods of time after the end of stimulation, dying away gradually. This die-away may be regarded as reflected in the fact that the longer the latent period the smaller the mechanical response (Fig. 4). The nature of the excitation in the coelenterate neuro-muscular system is as yet unclear, as is indeed the precise character of the events which are involved in such slow contractions as are developed by the circular muscle of *Calliactis*. Two views seem possible: one is that the slow contraction is the expression of a mechanically slow contraction of the individual muscle fibres, the other that these contractions are in the nature of prolonged tetani with the further implication that excitatory stimuli arrive constantly at the myoneural junctions during the course of the contraction. There appears to be no direct evidence for the former view. Horridge (1956) has, however, made direct observations of slow contractions in the radial muscle of *Cyanea* and reports that the 'sustained weak contraction of the whole muscle appears to be a combination of many irregular local contractions out of phase with one another', which would suggest that, in this case at least, the contraction is tetanic in character. Further, in the circular muscle of the pedal disk of *Calliactis* stimulation can accelerate relaxation (Fig. 5); this effect is most readily interpreted upon the assumptions that the contraction depends upon excitatory impulses continuously arriving at the myoneural junction and that their activity is inhibited by the release, following stimulation, of an inhibitory transmitter at the junction. Certainly there is no evidence that the muscle must already be in a state of contraction before inhibitory events can be effective for it has been shown (i) that intercalated stimuli, given before the development of a contraction, will prolong the latent period (Table 3); (ii) that stimulation applied just as a contraction is starting to develop will prevent its further development (Fig. 3); and (iii) that continuous stimulation will inhibit contractions over long periods of time (Fig. 1*b*).

It may then be suggested as a provisional hypothesis that, with the circular muscles of the pedal ring, electrical stimulation liberates a slowly destroyed inhibitory transmitter at the myoneural junction and produces at the same time a state of prolonged excitation within the nerve net of that region which is maintained by some self-excitatory mechanism such as that postulated by Young (1938) in *Sepia*. The interaction of these two influences is responsible for the remarkably long latent periods found in these preparations.

Experiments using pedal rings designed to see whether the thresholds for excitation and inhibition were distinct gave negative results. This is hardly surprising as it seems probable from experiments with *Metridium* (Batham & Pantin, 1954) that electrical excitation stimulates the specialized through-conducting system, either directly or by way of sensory structures, while what may, by analogy with medusae (Horridge, 1956), be referred to as the 'diffuse nerve net' is only indirectly stimulated. The properties which have been here ascribed to the 'diffuse nerve net' differ markedly from those already well attested for the through-conducting system; the observations of Batham (1956) show that in *Metridium canum* (Stuckey) these two systems are probably histologically distinct, and until detailed information on the structure of the 'diffuse' system is available there seems no profit in considering further the material basis of the phenomena which have been described above.

In regions oral to the foot the behaviour of the circular muscles is different. When an unstretched preparation is first used it behaves in a manner akin to a pedal ring, showing long latencies, but fairly rapidly the contractions become double (Fig. 6). The fact that the time to the development of the second part of the contraction remains approximately constant suggests that the changing form of the response is due mainly to the growing excitability of the preparation rather than to a diminishing inhibitory effect; if the latter occurred the latency of the second contraction would, on the basis of previous assumptions, be expected to fall. Although this second latency is very variable, is showed no definite downward trend in a series of fourteen experiments in which it was observed. The persistence of inhibitory action is further demonstrated by experiments such as that shown in Fig. 8*a*.

The reason for the growth of excitability with activity shown by adoral rings is not clear. It might possibly be due to an effect of stretching upon the muscle: the contractions recorded in Fig. 6 have been orientated so that the gradual stretching of the preparation may be seen. However, pedal rings also stretch in a similar manner, and although a double response may sometimes be obtained the magnitude of the first response is always small. The effect certainly cannot be due to the slow washing out of narcotic, for experiments by Dr D. M. Ross (personal communication) show that recovery of spontaneous activity in the circular muscle of *Calliactis* after a period of magnesium narcosis takes only a few hours; moreover, if a fresh mid-column preparation is permitted to be active for some hours until a clear-cut double response is obtained and then allowed to lie unstretched for 12 hr. in sea water, the response of the preparation is almost unchanged when it is again set up for recording and stimulated.

It is suggested that the difference in latency between pedal and mid-column rings lies in the relatively lower level of excitation in the former. But there is some evidence that the normally observed latencies of the order of 35 sec. which characterize mid-column preparations are also prolonged owing to the presence of an initial inhibitory effect. In a limited number of mid-column preparations initial latencies which lay between 6 and 10 sec. were found. In these cases there was a small initial contraction followed by a pause and then, after the more usual latency of about 35 sec., the shortening continued. The behaviour of these preparations recalled that of a mid-column preparation giving a typical double response, albeit the present event was on a shorter time scale. Unfortunately, lack of time made it impossible to study this phenomenon further, and it cannot be excluded that the first response of brief latency may be due to a direct effect of the stimulus upon the muscle tissue.

The second problem to be considered is the origin of the rhythmical spontaneous activity shown by these preparations of circular muscle. This property is not dependent upon the intactness of a ring, being shown as clearly by rings cut across and ligatured at both ends, and also by rings cut obliquely across the column so that no complete circle of muscle fibres remains.

The experiments illustrated in Figs. 8*b* and *c* show that following a contraction the response to a test stimulus grows as the interval between standard and test stimuli lengthens, and that if this period is sufficiently prolonged an all but maximal shortening of the ring may follow a single stimulus. It has been suggested that this effect is due to the presence of an inhibition whose slow decay is reflected in the gradual development of excitability in the preparation. Clearly such a mechanism could produce a rhythmical series of contractions if it is assumed that a naturally occurring spontaneous contraction arises from excitation within the nerve net, but that, in its occurrence, it produces a prolonged inhibition which prevents the development of a further contraction until it has died away. Such an explanation receives support from the results shown in Fig. 7*a*, where it will be seen that the extent of the shortening to intercalated stimuli increases as the time between the start of a spontaneous contraction and the stimulus is increased. Indeed there is a very striking similarity between Figs. 7*a* and 8*b*, albeit one shows spontaneous activity and the other an artificially imposed rhythm. The growth of relative excitability with time can also be seen in Fig. 8*c* where small spontaneous contractions occur when the time interval between standard and test stimulus is long.

It is therefore suggested that the rhythmical contractions arise from an inhibitory feed-back generated in some manner by the contraction of the circular muscles themselves. Such an interpretation is in keeping with the results obtained when studying the effect of intercalated stimuli upon the spontaneous activity rhythm (Fig. 7). It was in this case suggested that where the intercalated stimulus is purely excitatory in action the normal rhythm of activity is not upset, from which it would follow that the pace-maker is not integral with the system excited by electrical stimulation. If indeed the rhythm is controlled by a prolonged inhibition at the myoneural junctions normally initiated in some manner by the spontaneous contractions themselves, this is the type of result which would be expected to follow.

There are various ways in which this feed-back could originate. The first is that there is a simple reciprocity between the circular and parietal muscles of the column. A few preliminary experiments were made with preparations consisting of a ring of subsphincter or of low mid-column to which a tail of the column remained attached. Recordings of the activity of the ring and of the tail simultaneously gave no indication of any reciprocity in the activity of circular and parietal muscles. The second possibility is that there is some type of proprioceptor in the column which, stimulated by a contraction of the circular muscle, excites the inhibitory mechanism in the anemone. Evidence in favour of such an assumption cannot be obtained with the methods used in these experiments, for it would depend upon being able to stimulate the muscles to contract without also stimulating the inhibitory mechanism; this cannot be achieved with existing techniques of electrical stimulation. The final possibility is that the excitatory influences which cause the circular muscles to contract also excite the inhibitory mechanism so as to bring the contraction to a stop. Again no arguments for or against such an hypothesis can be advanced upon the basis of the present evidence and technique.

Mention must be made of the great variation in character of the pattern of activity of different preparations. An analysis of the performance of thirty-three preparations has been made; these have been divided into three classes—those showing complete inhibition of activity when continuously stimulated, those showing a partial but definite contraction and those showing a strong and sustained contraction. These three categories correspond to Fig. 1*b*, *c* and *a*, respectively. In nine out of twelve preparations assigned to the first group the spontaneous pattern was irregular, while in the three cases in which there was a regular rhythm it was slow with one contraction every 15–20 min. The second and third categories both included a few preparations showing an irregular pattern; four out of eleven in the second and three out of ten in the third. When the rhythm was regular its frequency was relatively high with a mean interval of 11 min. in both categories. It is clear that those preparations which more readily display inhibition also show a slower rhythm—a result which would follow from the hypothesis already elaborated.

In some preparations it is clear that complex rhythms arise because the circular muscles of the preparation are not all contracting together; the muscle has fragmented into a series of independent regions which may each have a slightly different rhythm and their resulting activity will be a series of contractions of varying extent and without any pattern obvious to casual inspection.

The hypothesis which has been elaborated to account for the origin of rhythmical spontaneous activity in the circular muscles of *Calliactis* is broadly similar to that which has been suggested as an explanation of similar events in the pharyngeal retractor muscle of *Cucumaria* (Pople & Ewer, 1958). In both cases it has been assumed that the slow contraction, which has a very similar time course in both preparations, is a tetanic contraction; it is possible to show in *Cucumaria* that stretching of the pharyngeal longitudinal muscle, which produces an inhibition, results in an acceleration of the rate of relaxation of the retractor muscle following a spontaneous contraction, just as electrical stimulation will accelerate relaxation in *Calliactis* circular muscle (Fig. 5). In both cases it is suggested that an inhibitory feed-back mechanism of prolonged activity is involved and in both it is suggested that this inhibition acts in opposition to a general excitatory input. In both cases evidence for inhibitory phenomena and for the existence of balances between excitatory and inhibitory influences has been presented. But while in *Cucumaria* it has been suggested that the inhibitory event lies within the motor complex proper to the pharyngeal retractor muscle, in *Calliactis* the evidence suggests that this may lie at the level of the myoneural junction. However, the results presented here cannot exclude the possibility that the spontaneous activity of the circular muscles of *Calliactis* is myogenic in origin and that electrical excitation stimulates extrinsic excitatory and inhibitory nerves just as the nerve supply to the myogenic heart of a vertebrate can control its rhythm.

SUMMARY

1. The responses to electrical stimulation of isolated rings of the column and pedal disk of *Calliactis* are described. Such rings make slow spontaneous contractions which are frequently rhythmical, the interval between contractions normally being 7–20 min.

2. Continuous low-frequency stimulation inhibits spontaneous activity of rings from the pedal disk and also of fresh rings from more adoral regions of the column. Older rings from the mid-column respond to such stimulation by a tetanic contraction.

3. The latency of response to electrical stimulation of pedal rings is of the order of 120 sec. This latency is not affected by stimulation frequency but is prolonged by increase in the number of shocks applied.

4. Stimulation of a pedal ring at the onset of a contraction prevents the further development of this contraction, while stimulation as a contraction reaches its maximum is followed by more rapid relaxation than in unstimulated controls.

5. Mid-column rings when freshly prepared show a latency of the order of 120 sec. As the preparation ages, a double response to stimulation appears; the first response has a latency of about 30–40 sec. and presently becomes the only type of response shown.

6. If two sets of stimuli are applied to a mid-column ring, the magnitude of response to the second set increases as the time between stimulations increases. With long intervals an almost total contraction is obtained in response to a single shock.

7. The effect of intercalated stimuli upon the rhythm of spontaneous activity is studied. The effect is very variable and it is suggested that this is the result of electrical stimulation having both an excitatory and an inhibitory effect.

8. The very long latent periods characteristic of pedal rings and the rhythmic activity of these preparations are interpreted as interactions of excitation and inhibition.

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TRANSMISSION THROUGH THE LAST ABDOMINAL GANGLION OF THE DRAGONFLY NYMPH, *ANAX IMPERATOR*

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INTRODUCTION

Mechanical stimulation of the abdomen or anal appendages of a dragonfly nymph generally elicits a characteristic escape response. The legs are drawn into the side of the body, water is rapidly ejected from the rectal chamber and the animal is propelled forwards (Tonner, 1936; Snodgrass, 1954; Hughes, 1958). The movement is analogous to that of the evasion response of the cockroach and suggests a similar approach to the problem in terms of transmission in the abdominal cord and through the last abdominal ganglion (Pumphrey & Rawdon-Smith, 1937; Roeder, 1948).

Synaptic transmission in insects has received relatively little consideration in comparison with the crustacea, and the dragonfly nymph provides several advantages for such a study. It is a large aquatic insect and the histology of both the central and peripheral nervous system has been studied in some detail (Zawarzin, 1924; Rogosina, 1928). Hughes (1953) showed that the fibres of the ventral nerve cord of late instar nymphs of *Anax imperator* range up to 16 μ in diameter and are comparable to the giant fibres of the cockroach or locust (Roeder, 1948; Cook, 1951). He further demonstrated that stimulation of the anal appendages is followed by bursts of activity in which large spikes are prominent in the nerve cord. The present paper is a more detailed analysis of the escape response with particular emphasis on the peripheral connexions of the 'giant' fibres and the characteristics of transmission through the last abdominal ganglion. The properties of the latter are shown to be of more general interest in demonstrating the existence of a simple segmental reflex in an invertebrate ganglion.

MATERIAL AND METHODS

Last instar nymphs of *Anax imperator* were kept in aerated tanks at a temperature of 10–15° C. They were fed regularly on blowfly larvae but starved just prior to an experiment. Intact nymphs were pinned in a wax dissecting dish containing insect Ringer (7·5 g. NaCl, 0·1 g. KCl, 0·2 g. CaCl, 0·2 g. NaHCO, distilled H_2 1000 ml.). A minimum of exoskeleton was removed in order to expose the ventral nerve cord and care was taken to ensure that the tracheal system to the ganglia remained intact. It was rarely necessary to remove any muscles except when dissecting the paraproct nerves for electrical stimulation. Breathing continued for 2–3 hr. and aeration was aided by injecting aerated water into the rectal chamber with a fine pipette. Preparations remained viable for 5 hr. or more.

The twin-channel recording system consisted of Tektronix Type 112 and Grass P4 preamplifiers and a Cossor double-beam oscilloscope. Two pairs of tapered silver/silver chloride electrodes were used for leading off activity. Stimulating electrodes were also of silver/silver chloride and pulses were provided by a rectangular pulse generator. Fine-trimmed paint brushes and tapered needles were used for mechanical stimulation of the abdomen and anal appendages.

RESULTS

Innervation of the terminal abdominal segments

The ventral nerve cord of the dragonfly nymph comprises three thoracic and seven abdominal ganglia. Zawarzin's (1924) classical studies on *Aeshna* are largely concerned with the organization of the second thoracic and fourth abdominal ganglia. Similarly the work of Rogosina (1928) is restricted to the distribution of nerves from the sixth abdominal ganglion and there is little reference to the seventh. It is therefore relevant, as a background to the physiology involved, briefly to describe the innervation of the terminal abdominal segments and anal appendages from the last abdominal ganglion. For this purpose animals were dissected *in vivo* and the nervous system stained with Rongalit methylene blue.

The seventh ganglion is larger than those of the other abdominal segments, innervates a greater area and possesses five pairs of lateral nerves (N 1–N 5, Fig. 1). Three pairs run laterally, innervate segment 8 and are comparable in their distribution to the abdominal roots of *Aeshna* (Rogosina, 1928). The remaining two pairs (N 4, N 5) are stout nerves running posteriorly to innervate segments 9 and 10 and the anal appendages. Physiologically it was shown that roots N 1, N 2, N 4 and N 5 are mixed nerves but N 3 contains motor fibres only, an observation that is supported by Zawarzin's histological study of the fourth ganglion.

The general distribution of nerves from the seventh ganglion is shown in Fig. 1; considerable variability occurs in the terminal branches. N 1 is a stout nerve with motor branches to the longitudinal sternal muscles, dorso-ventral oblique segmental muscles and the longitudinal tergal muscles (nomenclature from Whedon, 1918). It receives sensory fibres from the anterior part of the sternite, the pleurite, including lateral spine, and the tergite. Fibres from the stretch receptors have been demonstrated oscillographically in this first nerve which therefore resembles those of the other abdominal ganglia (Finlayson & Lowenstein, 1958). N 2 is a smaller nerve and appears to be composed of a larger proportion of sensory fibres than N 1. It branches freely ventrally and supplies the longitudinal sternal muscles and dorso-ventral segmental muscles. Sensory fibres from most of the sternite and part of the tergite run in this second root. N 3 is a very fine nerve running at first caudally, then turning laterally to supply the longitudinal sternal muscles, dorso-ventral oblique and segmental muscles.

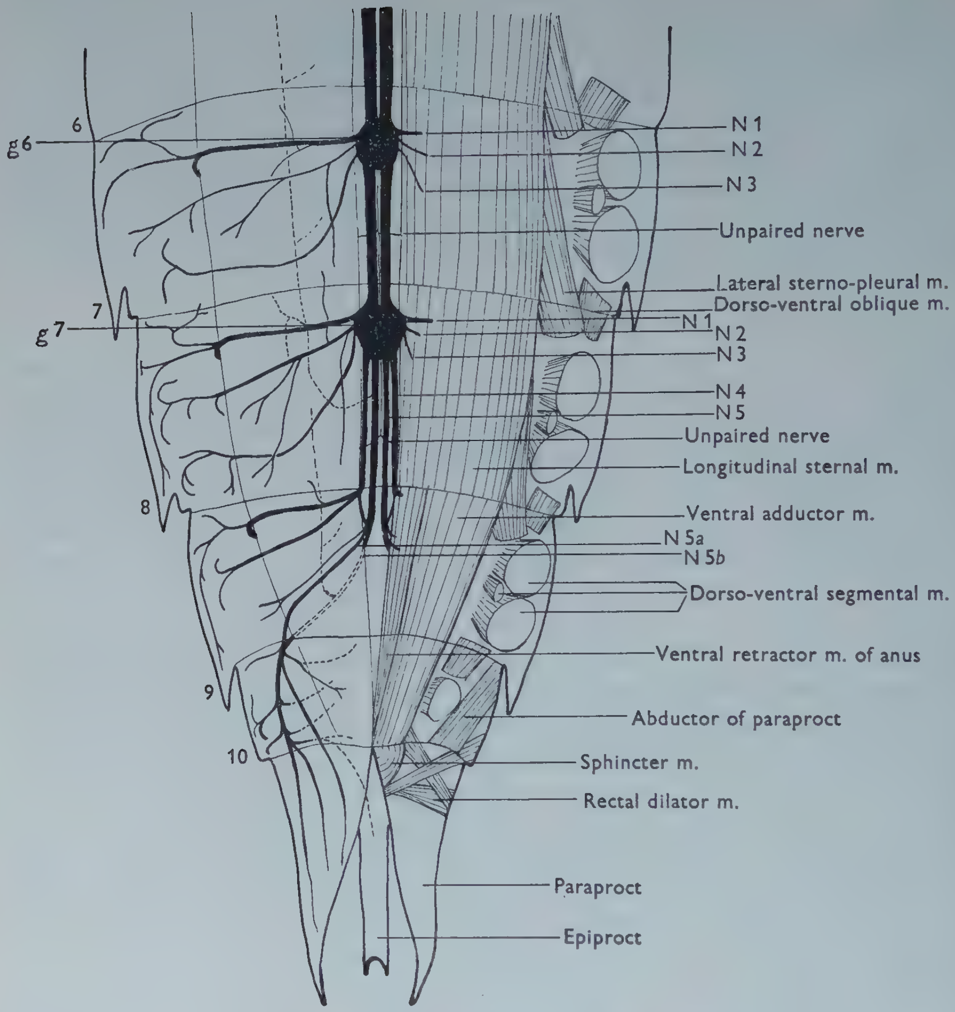

Fig. 1. Diagram of the terminal abdominal segments of *Anax imperator* nymph showing the major muscles and innervation from the sixth and seventh ganglia. Ventral view; m, muscle, g, ganglion, N, segmental nerve.

The fourth nerve, N 4, innervates segment 9 and appears to be rather similar in its distribution to N 1. It supplies the longitudinal sternal muscles, dorso-ventral oblique segmental muscle and longitudinal tergal muscles. An additional branch sends motor fibres to the dorso-ventral segmental muscles. N 4 receives sensory fibres from the sternite, pleurite and tergite of segment 9 and from stretch receptors. N 5 is the largest root and as shown by sections (Holmes silver, iron haematoxylin) contains the greatest number of fibres, which is correlated with its distribution to part of segment 9, segment 10, the anal appendages and respiratory chamber. It runs parallel to N 4 for some distance and often in the same sheath, then divides into two branches which pass over and under the longitudinal muscles and fuse at the anterior edge of segment 10 (*a* and *b*, Fig. 1). In segment 10 longitudinal sternal and tergal muscles, dorso-ventral oblique muscles, abductors of the paraprocts, retractor muscles of the anus, rectal and sphincter muscles are all supplied by motor fibres in branches of N 5. Large branches also run to the respiratory chamber extending as far forward as segment 5; these appear to be purely motor in function. N 5 receives sensory fibres from the sternite of segment 9, sternite, pleurite and tergite of segment 10, paraprocts, lateral and median spines.

It can be seen from this brief description that the last abdominal ganglion of *Anax* is concerned with the reception and distribution of information from a relatively large area of the body. In this respect the distribution of its paired roots is more extensive than that of the last ganglion of the cockroach or locust (Roeder, 1948; Cook, 1951). Recording from the peripheral ends of nerves of the seventh ganglion showed that the sensory representation deduced physiologically supports the anatomical evidence. Similar peripheral distribution was shown to occur in nerves of the other abdominal ganglia, where responses were obtained from stimulation of the same segment but not adjacent segments.

Responses to mechanical stimulation

Responses recorded from the ventral nerve cord of the dragonfly nymph have to be studied against a background of spontaneous activity and breathing discharges. The latter appeared to be of two different patterns accompanying the two types of breathing (Tonner, 1936). Shallow breathing was often characterized by rhythmic bursts from repetitively firing fibres, whilst in gulping breathing expiration was typically accompanied by a prominent burst of higher frequency in many more fibres. Gulping breathing and the response to mechanical stimulation of the paraprocts appeared to be due to a very similar sequence of movements of the abdomen, paraprocts and anal sphincter. These movements are the same as those involved in swimming and have been described by Hughes (1958). Briefly stimulation of tactile hairs on the inner surface of the paraprocts or of the anal sphincter elicited a rapid contraction of the abdominal muscles and of the intrinsic gut muscles, opening of the anal sphincter, divergence of the paraprocts and ejection of water from the rectal chamber. Simultaneously all six legs were drawn into the side of the body. This evasive response was not always seen on stimulation and was sometimes observed on touching other parts of the body.

Electrodes placed on a connective between ganglia 6 and 7 and the ipsilateral N 5 showed that the pattern of nervous activity associated with gulping breathing and the escape response had similar characteristics (Fig. 2). Both pre- and post-ganglionic recordings showed a large burst of activity in which large spikes were prominent. In the cord the response typically began in large fibres which rapidly increased in frequency as more units became active and often ended in a slow discharge. The burst sometimes lasted as long as 0·5–1·0 sec. The present results confirmed those of Hughes (1953) in showing that the large spikes accompanying stimulation of the anal appendages travelled through the abdominal and thoracic ganglia without synapsing. The ascending barrage excited efferents in all the abdominal and thoracic roots, as recording from the central ends of the segmental nerves showed that motor discharges accompanied gulping breathing or the events associated with mechanical stimulation. Stimulation of the head gave fewer large spikes many of which did not traverse the length of the cord. An attempt was made to simplify the post-ganglionic response from the last ganglion by fine dissection following de-sheathing of the connectives. This has so far not been successful owing to poor survival of the preparations.

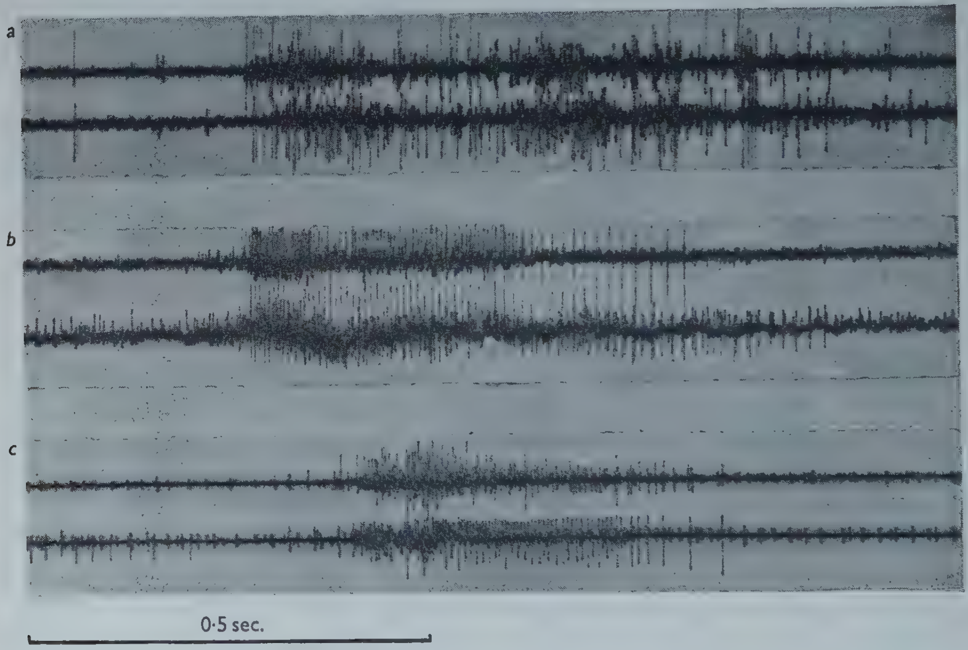

Fig. 2. Records of the nervous activity arising from mechanical stimulation of the paraprocts in *Anax* nymph. (*a*) Large spikes recorded between ganglia 6 and 7 (upper trace) and thoracic ganglia 2 and 3 (lower trace). (*b*) Synchronous firing of units in the ipsilateral N 5 (upper trace) and ipsilateral connective (lower trace). (*c*) Efferent spikes in the ipsilateral N 5 (upper trace) and the cut proximal end of the contralateral N 5 (lower trace). Time scale 0·5 sec.

Recording from N 5 showed that the large burst of activity on stimulation was correlated with the abrupt opening of the anal sphincter, suggesting that part of the activity was in efferent neurons. This was shown to be the case by placing the other pair of electrodes on the cut central end of the contralateral N 5, when large discharge occurred in both nerves on stimulation of the anal appendages (Fig. 2). Contraction of the sphincter was asymmetrical under these conditions. Comparison of the two recordings showed that most of the activity in the ipsilateral N 5 was efferent and that the afferent spikes were in small fibres only. Recording from the peripheral end of N 5 supported this observation, as the sensory inflow from tactile receptors was seen to be only a relatively small response. The large efferent bursts were unaltered by severing all inputs to the ganglion and appeared as a much more definite and predictable reflex than that described in the crayfish caudal ganglion (Fielden, 1960). Mechanical stimulation of the paraprocts therefore gives rise to a small afferent inflow to the last ganglion which excites efferent neurons in both ipsi- and contralateral N 5's. Recording from other nerves showed that this inflow also excited interneurons in ipsi- and contralateral N 1, N 2, N 3 and N 4 and the unpaired nerves. Occasionally the response in N 5 and a connective showed a 1:1 relationship of large spikes on either side of the ganglion, which was therefore due to synchronous firing of efferent fibres in the segmental nerves and interneurons in the connective (Fig. 2). Similar synchronous firing was sometimes noted in the efferent spikes in the two N 5's or from N 5 and the ipsilateral N 4 and in gulping breathing. It seems that the latter is due to the excitation of the same groups of neurons by some small afferent source as in mechanical stimulation.

It was impossible to decide from recording the nervous activity due to mechanical stimulation whether fibres pass through the last ganglion from receptors in the terminal abdominal segments, or whether afferents always synapse with ascending fibres in the ganglion. Electrical stimulation was therefore used to clarify this point and to study the segmental reflex in more detail.

Responses to electrical stimulation

Motor responses. The results confirmed the previous observations (Hughes, 1953) that stimulation of the abdominal nerve cord produces a precisely similar response to that seen on touching the paraprocts. The characteristic leg movement has a strict frequency dependence—the legs are drawn into the side of the body only at frequencies above 20–30/sec., but some movement of the abdominal muscles, intrinsic gut muscles and paraprocts occurs in a 1:1 manner on stimulation. It has now been established that stimulation of N 5 of the last abdominal ganglion also elicits the evasive response and movement of both contra- and ipsilateral legs at frequencies of 20–30/sec. Stimulation of N 4 produced a similar response, but this was less predictable and often not shown by the contralateral legs. Movement of the rectal chamber was seen only if N 5 remained intact.

A markedly different response was seen on stimulation of the ventral nerve cord anterior to the prothoracic ganglion. The legs in this case were bent in the grasping position, the response being similar to that seen on seizing the head. These responses immediately suggest a study of the transmission involved to the segmental nerves of the thoracic and abdominal ganglia.

Neuronal pathways in the last ganglion

The results of tracing pathways using electrical stimulation are summarized in Table 1. They illustrate still further the diffuse nature of the neuronal connexions in the ganglion. Diffuse connexions appear to be a characteristic property of the dragonfly nervous system as similar responses were obtained from the other

Table 1. *Results of tracing neuronal connexions in the last abdominal ganglion of* Anax *nymph*

	Response												
	Ipsilateral							Contralateral					
Stimulation	N 1	N 2	N 3	N 4	N 5	unp.	conn.	N 1	N 2	N 3	N 4	N 5	conn.
N 1	·	·	·	+	+	·	+	+	·	·	+	+	+
N 2	·	·	·	+	+	·	+	·	·	·	·	·	+
N 3	·	·	·	–	–	·	–	·	·	·	–	–	–
N 4	+	+	+	·	+	+	+	+	·	+	+	+	+
N 5	+	+	+	+	+	+	+	+	+	+	+	+	+
conn.	+	+	+	+	+	+	·	+	+	+	+	+	+

+ response on stimulation; – no response; N 1–N 5 segmental nerves; conn. connective; unp. unpaired nerve.

abdominal ganglia. Their existence is supported by Zawarzin's (1924) histological work on the fourth ganglion of *Aeshna*. Some of the pathways showed distinct synaptic properties in terms of thresholds, delays and transmission frequencies, while others indicated that 'through' fibres were present. In many preparations the same group of neurons could be recognized in a barrage excited by stimulation of several different inputs to the ganglion. For example, in one rather clear experiment as far as could be identified an identical group of efferent fibres in N 4 were excited by stimulation of the ipsilateral connective, N 1, N 2 and N 5, and the contralateral connective, N 1, N 4 and N 5.

Stimulation of the segmental nerves of the last ganglion

Stimulation of N 5 and recording from electrodes proximally on N 5 and on the ipsilateral connective clearly showed the two-component nature of the response seen on mechanical stimulation. Fig. 3*c* illustrates a typical result. Stimulation at position 1 gave an afferent burst, recorded at 2, which excited two post-ganglionic responses: one in the connective recorded at 3 and the other in the same N 5 recorded at 2. These post-synaptic responses possessed different thresholds, delays and frequency relationships. The effects of increasing intensity of stimulation are shown in the series 3 *a*–*c* and resulted in the following pattern of response.

(*a*) At lowest intensity afferent fibres only were excited. These conducted at a velocity of 1·5–2·5 m/sec. and the spikes appeared to be in small fibres.

(*b*) Slightly raising the intensity increased the afferent volley and elicited a post-ganglionic response in the connective. This showed a ganglionic delay that was relatively constant in a given preparation but ranged from 2·0 to 4·5 msec. in different preparations. The response in the connective was difficult to characterize as it was composed of a large number of often compound spikes of similar size and threshold. The larger spikes, excited at low intensity and with a short delay, travelled the length of the cord at a velocity of 3·8–4·5 m./sec. apparently without synapsing.

(*c*) A further increase in intensity excited the efferent neurons of N 5. It also resulted in stimulation of units with a longer delay in the connectives and often shortened the delay for some of the larger spikes by as much as 0·8 msec. The post-synaptic response in N 5 is of particular interest as it demonstrates the existence of a segmental reflex in the seventh ganglion. The motor neurons invariably possessed a higher threshold and longer, more variable delays (3·5–8·0 msec.) than did the units responding in the connectives. The spikes travelled at a velocity of 2·5–3·0 m./sec. in the paraproct nerves and were larger than those in the sensory fibres. Several of the units appeared to fire repetitively to a single afferent shock in some preparations even when all inputs to the ganglion were severed. Repetitive

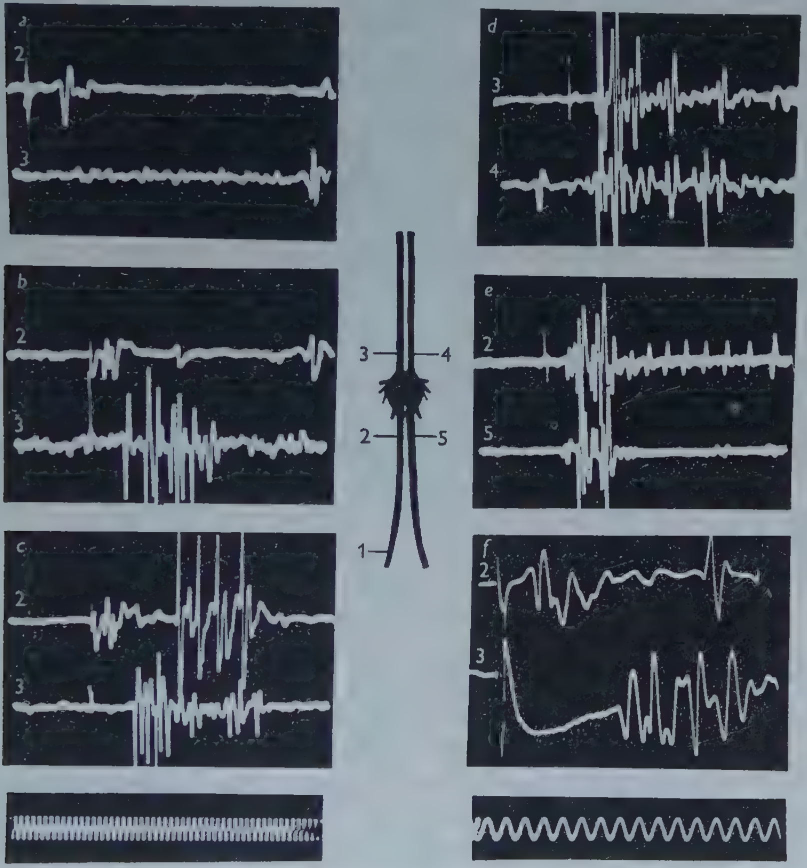

Fig. 3. Activity arising from electrical stimulation in *Anax* nymph (*a*–*c*). Series showing the effect of increasing the intensity of stimulation of a paraproct nerve N 5; upper recording, electrodes proximally on the same N 5, lower, electrodes on the ipsilateral connective. (*a*) Afferent response only. (*b*) Post-synaptic response in the connective. (*c*) Both post-synaptic responses. (*d*) Recording from ipsilateral (upper) and contralateral (lower) connectives on stimulation of N 5. (*e*) Recording from ipsilateral (upper) and contralateral (lower) N 5's on stimulation of a connective. (*f*) Both post-synaptic responses as in (*c*) faster sweep, different preparation. Electrode positions numbered on traces and shown in the diagram. Time scale msec.

firing was also seen in large units in the connectives similar to those occurring on mechanical stimulation.

Increasing the frequency of stimulation resulted in the post-synaptic units of N 5 dropping out at frequencies of 30–40/sec. The majority of units in the connectives were blocked at similar frequencies, but some of the larger spikes persisted to 50–70/sec. and occasionally higher. The repetitive spikes in the paraproct nerves and connectives ceased at frequencies of 5–10/sec. On the basis of these observations it is concluded that afferent fibres from the anal appendages synapse with several large fibres which ascend the cord. No evidence was found for afferent fibres entering the last ganglion from the paraproct nerves without synapsing, and the preparation therefore resembled that of the cockroach (Roeder, 1948; Hess, 1958). A comparison of transmission with that through the last abdominal ganglion of the cockroach gave no evidence for the adapted state described for the giant-fibre synapses by Pumphrey & Rawdon-Smith (1937).

The response in the contralateral connective on stimulation of N 5 is shown in Fig. 3*d*. It appeared similar to that in the ipsilateral connective and in this respect differed from that of the cockroach (Roeder, 1948). It showed the same threshold, delay and frequency relationships and included spikes which travelled throughout the cord. Crossing of both ascending and descending barrages was shown to occur in the other abdominal and thoracic ganglia, where a difference in threshold and delay distinguished these synapsing contralateral fibres from the 'through' fibres of the ipsilateral side.

Observations on the other roots of the seventh ganglion showed that stimulation in all cases elicited a response in the connectives except for N 3 which is purely motor in function. Stimulation of N 4 excited a smaller barrage than did stimulation of N 5, but this included large spikes which were conducted straight through the other abdominal ganglia. Stimulation of N 1 revealed an interesting feature in that afferent fibres appeared to pass through the ganglion to the ipsilateral connective without interruption by a synapse. Spikes with a short delay (0·2–0·4 msec.) and following frequencies of more than 160/sec. were recorded in the connective. Similar responses were seen in the anterior connective following stimulation of a first nerve of the other abdominal ganglia. Histological evidence (Zawarzin, 1924) supports the conclusion that these are in 'through' afferent fibres comparable to those described in the roots of the crayfish abdominal ganglia (Hughes & Wiersma, 1960).

Stimulation of the connectives

Stimulation of a connective gave rise to responses in contra- and ipsilateral nerves of the last ganglion (Fig. 3*e*), and movements of the paraprocts and sphincter comparable to those seen on direct stimulation of N 5. These efferent synapses proved to be of particular interest as they showed a definite temporal summation or facilitation effect of increased frequency that was not seen at the afferent: ascending fibre synapses. With an intensity just subthreshold an increase of frequency produced an efferent burst in the fourth and fifth nerves in particular. At threshold intensity an increase in frequency to 10–30/sec. also excited more efferent units.

These effects were most obvious in older preparations, as they are in the 'adapted' state in the cockroach. Similar facilitation or summation was seen in the segmental nerves of the other abdominal ganglia, and was especially prominent in the thoracic nerves where a response was rarely seen at frequencies below 5/sec. even in fresh preparations. The first roots again showed a compound spike with a very short delay which is interpreted as due to antidromic excitation of direct afferent fibres. Responses in N 4 and N 5 often included units which fired repetitively to a single afferent shock (Fig. 3*e*).

DISCUSSION

The evasion response of the dragonfly nymph depends on neuronal pathways and connexions very similar to those of the cockroach. In the last abdominal ganglion afferent fibres from tactile receptors on the paraprocts synapse with large fibres which ascend the length of the cord and synapse with efferents in the thoracic and abdominal ganglia. Closer investigation of the thoracic ganglia may reveal synapses between the large fibres in the connectives entering and leaving the ganglia, but this seems unlikely on histological and physiological grounds. As in the cockroach, the degree and duration of the evasion response is dependent not only on the intensity of stimulation but on the characteristics of the synaptic junctions between (*a*) sensory neurons and ascending fibres, and (*b*) these fibres and efferents in the segmental nerves. There is evidence in the dragonfly that these two types of junction possess different properties comparable to those of the cockroach, which is the only insect in which synaptic transmission has yet been studied in any detail.

It is of interest to compare the 'giant' fibres system and its peripheral connexions in the dragonfly, cockroach and locust (Table 2). Although the systems appear very similar the functional significance of the pathway in the locust is not very obvious as the evasion response is absent (Cook, 1951). The response is of evident survival value in the cockroach and dragonfly. These are believed to differ, however, in the presence of more widespread afferent connexions to the 'giant' fibres in the dragonfly. In the latter the last abdominal ganglion appears to be of more localized importance than in the cockroach in view of its large motor output to the muscles of the posterior abdomen and rectum. Zawarzin (1924) has suggested that this ganglion is a composite of four which may be correlated with the large area supplied by the ganglion and the extremely diffuse connexions between its roots.

Transmission through the last ganglion and abdominal nerve cord is very similar in general pattern. There is evidence that many tactile afferents transmit to relatively few large fibres and a synapse exists in all cases. The shorter delays found by Roeder (1948) in the cockroach may be due to the comparatively larger size of the ascending giant fibres, or may indicate a monosynaptic pathway in comparison with a polysynaptic one in the dragonfly. The properties of the latter do not preclude the possibility that interneurons are present between afferents and ascending 'giants', but this seems unlikely. Comparative evidence from the crayfish caudal ganglion supports the suggestion that the larger fibre synapses have shorter delays (Fielden, 1960). Delays in both the dragonfly and cockroach shorten

Table 2. *Comparison of the 'giant' fibre system and its peripheral connexions in* Anax *nymph,* Periplaneta *and* Locusta

	Anax nymph	*Periplaneta*	*Locusta*
Size 'giant' fibres	12–16 μ*	20–45 μ†	8–15 μ‡
Number 'giant' fibres	6–7*	6–8†	4‡
Cond. velocity 'giants'	3·5–4·5 m./sec.	6–7 m./sec.†	3–4 m./sec.§
Connexions in last ganglion	Synapse with efferents from paraprocts	Each synapse with several afferents from cercae†	Synapse with afferents from cercae§
Properties of afferent synapse with 'giant' fibre			
(*a*) Delay	2·0–4·5 msec.	1·4–1·9 msec.†	2–3 msec.§
(*b*) Transmission frequency	50–60/sec.	70–100/sec.‖	70–80/sec §
(*c*) Facilitation	——	+‖	?
temporal summation	——	——†	?
Properties of synapse between 'giants' and efferents in thoracic nerves	Labile, fatigues, facilitation or temporal summation important	Labile, fatigues, temporal summation important†	?

* Hughes, 1953: † Roeder, 1948; ‡ Cook, 1951; § Fielden, unpublished observations; ‖ Pumphrey & Rawdon-Smith, 1937.

on increasing the intensity of stimulation. Similar shortening has been observed for single units in the crayfish ganglion where it has been suggested that the post-fibre has a graded longitudinal threshold for the several afferents synapsing on it (Preston & Kennedy, 1960).

The major physiological control of the evasion response in the cockroach appears to occur largely at the labile synapses between ascending fibres and motor efferents in the thoracic ganglia (Roeder, 1948). The same feature is seen in the dragonfly where the efferent responses, particularly in the thoracic nerves, are very frequency-dependent. Whether this frequency effect is one of facilitation or temporal summation or both was impossible to decide on the gross scale of the current recordings, and it is hoped that future work at the unit level will throw some light on this problem. A clear distinction between facilitation and temporal summation is not always made in the literature, but it seems that temporal summation does not occur at the sensory: giant synapses of the cockroach last ganglion (Roeder, 1948). Facilitation is seen at these synapses in the 'adapted' state, but was not observed in the dragonfly ganglion under the conditions of this study. A further property requiring analysis at the unit level is that of repetitive discharges from large cells in the connectives and roots. The only arthropod where this has been shown to occur at a 1:1 junction is that of efferents in the roots of the crayfish abdominal ganglia which fire repetitively to one shock in a single giant fibre (Wiersma, 1952). After-discharges have been described in the cockroach (Roeder, 1953), but here, as in the dragonfly, they may be due to multiple connexions with variable delays or the firing of several afferents on a single efferent or interneuron, and it would be of interest to find a junction in the insect analogous to that in the crayfish.

The reflex stimulation of motorneurons in the fifth roots of the *Anax* last abdominal ganglion has not been studied in any comparable preparation in the cockroach. Indeed information on the relationship between sensory and motor nerves is very meagre in insects and restricted almost entirely to the highly specialized giants. Segmental reflexes concerned with respiratory, locomotory and sexual activities have been described in many insects largely through observations on behavioural changes after removal of portions of the central nervous system. Pringle (1940) describes a locomotory reflex in the cockroach in which he was able to show the effects of changes in afferent activity on tonus and contraction in different leg muscles. But there has not yet been a detailed study using controlled electrical stimuli of the connexions between afferent and efferent fibres and the dragonfly isolated ganglion, and a fifth root therefore provides a preparation analogous to that of the vertebrate spinal cord. Zawarzin noted this in his histological work and drew a comparison between the dorsal and ventral roots of the dragonfly and the vertebrate. The present results seem to indicate that delays in the dragonfly reflex are more comparable to those of the mammalian polysynaptic pathway (Alvord & Fuortes, 1954), but transmission frequencies are lower (Eccles, 1957). Further work on this preparation should give information on such problems as facilitation, inhibition and summation in the insect central nervous system.

SUMMARY

1. In the last abdominal ganglion of the dragonfly nymph afferent fibres from tactile endings on the paraprocts synapse with large ascending fibres in the ventral nerve cord. The latter run the length of the cord and synapse with efferent fibres in the segmental nerves of the thoracic and abdominal ganglia. The evasion response is mediated by this pathway and can be elicited by electrical stimulation of the nerves from the paraprocts.

2. The synapses between tactile afferents and ascending fibres show delays of 2·0–4·5 msec. and transmit synchronously to frequencies of 50–60/sec. There is no evidence for facilitation at these synapses, but facilitation and/or temporal summation is of importance at the efferent synapses which appear more labile in their properties.

3. Stimulation of the paraproct nerves shows the existence of a reflex connexion between afferents and efferents of the same nerve comparable to that of the vertebrate spinal cord. This provides a preparation for a study of the relationship between sensory and motor neurons in the insect nervous system.

4. Transmission through the last abdominal ganglion and cord is compared with that in the cockroach and locust.

I wish to thank Dr G. M. Hughes for suggesting this problem and for his help and advice during the course of the work and the preparation of this manuscript. I am also grateful to the Medical Research Council under whose auspices the work was carried out.

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HEARING AND THE OCTOPUS STATOCYST

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A detailed study of the anatomy of the octopus statocyst by Young (1960) seemed to indicate that, although the maintenance of equilibrium was likely to be its most important function, the possibility of its participating in the function of hearing could not be ruled out. The work described here was an attempt to test this possibility.

The general problem of the function of invertebrate statocysts was reviewed by Lowenstein (1950) who pointed out that structural comparisons with the vertebrate labyrinth might be misleading and that direct experimental evidence was needed. Boycott (1960) has described the effects of unilateral and bilateral removal of statocysts on the maintenance of equilibrium in *Octopus*, and Wells (1960) has described the effects of the same procedure, with its consequent displacement of the retina, on learned discrimination.

METHODS

The work was done at the Stazione Zoologica in Naples and *Octopus vulgaris* Lamarck of about 300 g. mass were used. They were placed in pressed asbestos tanks 100 × 60 × 40 cm. deep (Wells & Wells, 1956) through which a continuous supply of aerated sea water was circulated.

The apparatus used for giving sound stimuli through the water consisted of a moving-iron telephone earpiece driven from an audio-oscillator. The earpiece was modified in the following way:

(*a*) The diaphragm-retaining ring was removed so as to present the maximum vibrational area to the water, and the diaphragm itself was sealed with 'Araldite' adhesive to the earpiece all round its outer edge; all screw heads in the surface of the earpiece were also sealed and filled with the same material.

(*b*) A hexagonal-headed brass bolt, 12 mm. diameter, 5 cm. long, was drilled axially so as to allow a screened twin-core, P.V.C.-covered cable to be threaded through with a tight fit. The outer covering was arranged to lie about 3 mm. back from the face of the hexagon head and the emergent twin wires and the screening were sealed in with 'Araldite' adhesive; the exit of the cable at the other end of the bolt was also sealed. The twin wires were connected to the telephone terminals and the screening connected to one of them.

(*c*) A hollow cylindrical mould whose diameter was about 1 cm. greater than that of the earpiece was made from cold-curing silicone rubber (Midland Silicones Ltd. K9161, Catalyst N9162). This material will allow the release of at least twenty

'Araldite' castings. The earpiece and brass bolt were then clamped above the mould and lowered so that the face of the diaphragm rested on three symmetrically arranged small spacers of P.V.C. each about $\frac{1}{2}$ mm. thick. 'Araldite D' to which had been added 8 % by weight of hardening catalyst was then poured into the mould to a level just above the hexagon head and its junction with the shank of the bolt. After about 18 hr. the mould was removed and the unit was ready for fastening to the tank. Fig. 1 shows the various stages of the above procedure. Five earpieces were modified in this way and withstood continuous total immersion in sea water for 3 weeks without breaking down.

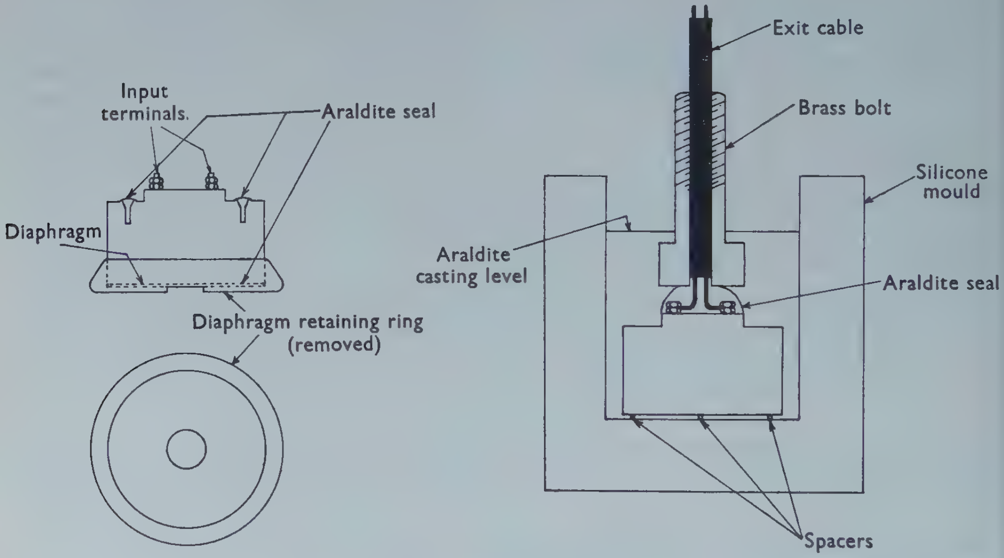


Fig. 1. Diagram showing how a telephone earpiece was modified to withstand continuous total immersion in sea water.

The earpieces were mounted singly in the tanks in the middle of the shorter side through a 12 mm. hole drilled at about half the depth of water (about 25 cm.) normally in the tank. A rubber washer and a flat brass washer on each side were sufficient to prevent leakage with only gentle tightening of the fixing nut; the latter was, however, very firmly tightened to prevent the octopus from turning—and thus loosening—the whole unit (this occurred several times during the installation of the units!).

Fig. 2 shows a block diagram of the electrical apparatus. The audio-oscillator was connected to the earpiece through a shorting key which was opened during the period required for the sound stimulus; for reasons which will be discussed later no attempt was made to limit the transients in the diaphragm at the beginning and end of the stimulus. Preliminary tests with a calibrated hydrophone in metal tanks rather smaller than those at Naples had shown that many resonances occurred over the range 50–30,000 cyc./sec.; these were due to the acoustic properties of the earpiece, mismatching at the diaphragm/water interface and reflexions and absorptions in the tank itself. To counteract this, in some measure, each unit was calibrated, before installation, in one of the tanks at Naples, and the oscillator output setting which gave maximum undistorted output over the range 50–30,000 cyc./sec. was recorded. The hydrophone used in these tests was suspended in the tank just in front of the octopus's 'home' (Boycott, 1954) and the output was amplified and displayed on an oscilloscope. Since the absolute calibration of the hydrophone was known it was therefore possible to estimate the effective hydrodynamic sound pressure at any frequency and to see at what level distortion occurred. Boundary effects at the walls of the tank were, however, large, so the figures for stimulus sound pressures were regarded only as giving an order of magnitude rather than an exact absolute figure. Further, this calibration was less useful than might have been expected since there was insufficient time to carry out training tests at more than two or three frequencies and, also, the results obtained were such that the distortion of the wave form from a pure sine curve was not likely to be of much importance. It was found that between 100 and 5000 cyc./sec. the wave-form of the hydrodynamic sound stimulus was undistorted in the neighbourhood of the hydrophone, provided a sound pressure of 17 dynes/cm.^{2} was not exceeded.

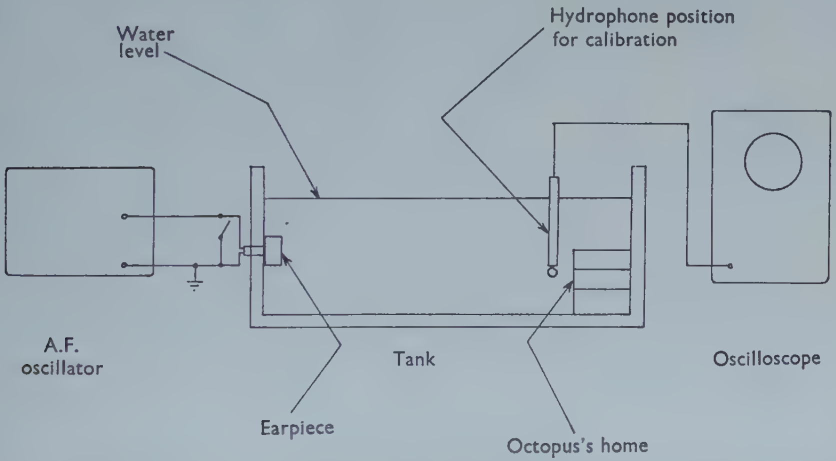


Fig. 2. Diagram showing the electrical connexions between the units which produced the sound stimulus in the tanks.

When the earpieces had been installed in the tanks the animals were trained to attack crabs and when the latencies of attack were about 5 sec., one of two further methods of training procedure and testing was adopted.

Training [illegible] attack

A crab was placed in the end of the tank opposite the octopus's home immediately after a short sound stimulus had been given. After five or six repetitions of this at about 10 sec. intervals the sound stimulus was given alone and the animal's response was noted.

Training to attack with discrimination

First, a 10 V. a.c. electric shock was given to the octopus in its home after a 10 sec. sound stimulus. Then, after 15 min., the two tests which constituted the training were begun. They consisted of presenting a crab, either alone or preceded by a 10 sec. sound stimulus. In the first case the octopus was allowed to capture—and eat—the crab; in the second case the crab was withdrawn and, in some experiments, an electric shock was given. The two presentations of the crab were repeated at intervals varying from 5 to 30 min. though in any one experiment the interval between tests was constant. In all cases the measured parameter was the latency of attack by the octopus after the presentation of the crab.

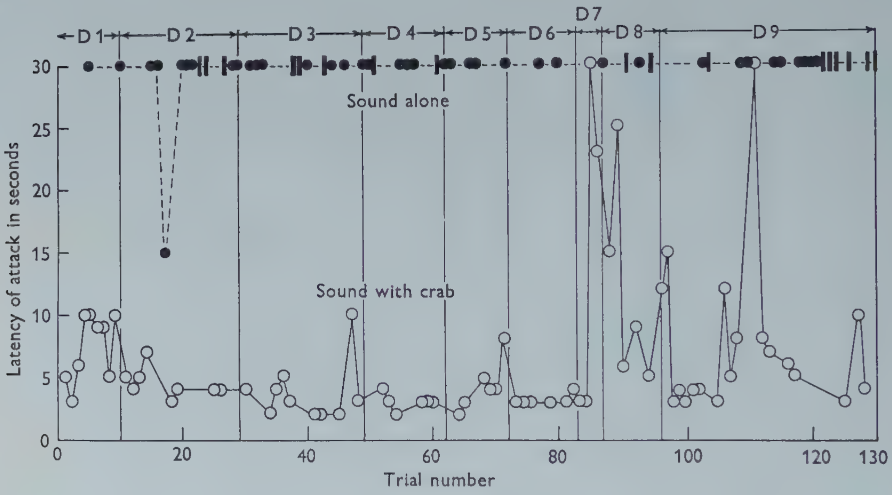


Fig. 3. Results of trials in training to attack. Abscissa: trial number. Ordinate: latency of attack in sec. Open circles: latency when crab given. Filled circles: latency when sound given alone. Solid vertical bars: movement in or slight emergence from home of octopus, usually just after the sound was switched off.

RESULTS

Training to attack

In these experiments three different frequencies—100, 900 and 2000 cyc./sec.—were used both with the same and with different animals on separate occasions; Fig. 3 shows the results from trials with one animal over a period of 9 days. On days 4 and 5 the frequency used was 100 and 2000 cyc./sec., respectively, but on all other occasions it was 900 cyc./sec. The trials were 5 min. apart and the sound was given for either 10 sec.—after which the crab was presented—or for 30 or 60 sec. alone. The 60 sec. period of sound was used on the last 3 days in case responses of more than 30 sec. latency had been missed in the previous tests. Open circles in the figure represent attack latencies when the crab was given; filled circles latencies with the sound alone, 30 sec. being the absolute limit of time measurement.

There was no evidence in these experiments to show that sound conditioning had occurred. It may be seen from the figure that in a total of 130 trials there was only one positive attack out of a possible fifty-seven occasions on which the sound alone was used. There were, however, seventeen occasions (shown as thick vertical bars in the figure) on which the animal showed some movement in its home or emerged slowly from it, usually after the sound was switched off, but none of these movements was comparable with the behaviour of the octopus when it was attacking a crab.

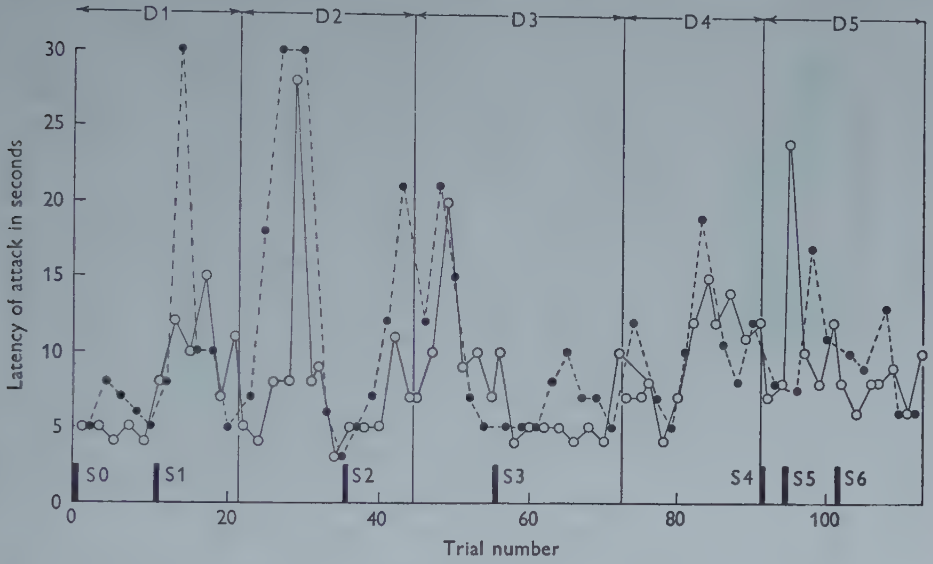
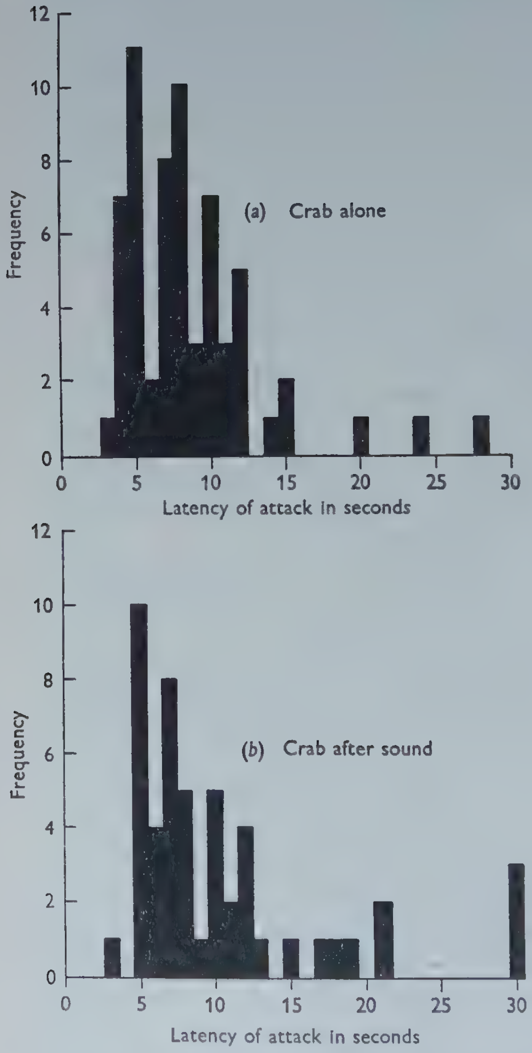


Fig. 4. Results of trials in training to attack with discrimination. Abscissa: trial number. Ordinate: latency of attack in sec. Open circles: latency when crab given alone. Filled circles: latency when crab given after 10 sec. sound. Short thick ordinates (S0–S6) represent 10 V. a.c. shocks given to octopus.

Training to attack with discrimination

Most of these experiments were done with a sound frequency of 100 cyc./sec. and Fig. 4 shows the results of trials on one animal over 5 days. The test intervals were 5 min. and the sound, when used, was given for 10 sec. In the figure the short thick ordinates signify when shocks were given to the animal in its home after a 10 sec. period of sound. No other shocks were given; if an attack was made when the crab was presented after the 10 sec. sound stimulus it was simply withdrawn before it could be eaten. The open circles joined by a continuous line represent attack latencies when the crab was given alone, and the filled circles joined by a broken line represent the latencies when the crab was given after 10 sec. of sound. It may be seen that the graph defined by the filled circles tends to lie above that defined by the open circles; if good discrimination were being performed by the octopus this separation of the two graphs would be more distinct.

Fig. 5 shows histograms of the attack latencies for the two forms of presentation and the inset Table gives the results of *t*-test calculations; these are given for the whole 5-day period and for the periods between the shocks (See Fig. 4). It may be seen that the mean latency of attack when the crab was preceded by the sound was greater than that without the sound except for one instance; but there was only one occasion when the difference of these means was significant ($P = 0{\cdot}05$). The fact that the distribution is truncated (since the upper limit of measurement was 30 sec.) does not significantly affect the calculations.



Test interval	$\mu_b-\mu_a$	n	t	$t(P = 0{\cdot}05)$
D_{total}	1·7	111	1·62	2·00
S0–S1	1·4	8	3·36	2·31
S1–S2	4·6	23	1·35	2·07
S2–S3	3·0	18	1·26	2·10
S3–S4	0·5	34	0·40	2·04
S4–S6	−0·8	8	0·22	2·31
S6–$D5_{end}$	1·0	10	0·97	2·23

$\mu_b-\mu_a$ = difference of mean latencies,
n = degrees of freedom.

Fig. 5. Histograms showing frequencies of latencies of attack when: (*a*) crab was presented alone, (*b*) crab was presented after 10 sec. of sound. The results are a total for one octopus over a 5-day period. (Inset Table shows *t*-test calculations for the whole interval and for intervals between shocks.)

DISCUSSION

It is quite clear that, on the evidence presented here, there is little justification for inferring that the octopus's behaviour was modified by the presence of sound waves, regardless of whether or not the statocysts were involved. It remains an open question as to whether the results represent the true sensory situation or are a consequence of the particular experimental methods used.

Before a fully satisfactory investigation of any possible sensory faculty of an animal can be made it is important to know what background level of stimulus for that faculty exists in the normal habitat of the animal and, further, what are the physical limits of the naturally experienced stimuli. In this case, therefore, we would like to know what background noise exists in the octopus's normal environment and what the amplitude and frequency limits of all possible auditory stimuli may be. Since this is at present unknown, the selection of test frequencies (for example) can be no more than arbitrary, although some clues may exist (Dijkgraaf, 1952). It is unlikely that the frequencies used in the present work had much bearing on the negativity of the results, since distortion (and, thus, many harmonics) occurred at sound pressures greater than 17 dynes/cm.^{2} (see Methods) and this figure was often exceeded; had the results been more positive, pure tones would have been important in estimating the frequency *range* of hearing.

In this context the definition of 'hearing' is of some consequence; Pumphrey (1940) has considered this in relation to insects and his comments are generally applicable although his definition of hearing excluded water-borne sounds. An important point which he made was that the separation of sound and tactile senses was 'based on the intensity factor' and was arbitrary. With this in mind there is no reason why we should not define hearing in marine animals as occurring when the animal is 'demonstratively responsive to sound' in this case sound being defined as low-intensity hydrodynamic disturbances.

The sound pressure used in this work was many times greater than that giving human threshold of hearing, but Fraser & Purves (1960) have shown, in a discussion on hearing in Cetacea, that the place of pressure in the response of terrestrial animals to sound is taken by displacement in marine animals. We can assess the adequacy of the pressures used here by noting that for any medium, and for a given sound intensity and frequency, the sound pressure and amplitude are connected by the equation (Pumphrey, 1940)

$$x = kp/\rho c,$$

where x = amplitude of sound, p = pressure of sound, ρ = density of medium, c = velocity of sound in the medium, k = constant. So for air $x_A = k_A p_A/\rho_A c_A$, and for water $x_w = k_w p_w/\rho_w c_w$, where the suffices refer to the appropriate medium. Dividing one equation by the other gives

$$x_A/x_w = (p_A/p_w)\times(\rho_w c_w/\rho_A c_A), \qquad (1)$$

since $k_A = k_w$ provided the sound is of the same frequency and intensity in either medium. Now $\rho_w c_w/\rho_A c_A = 3700$ approx. (Vigoureux, 1960) and, in these experiments, the minimum hydrodynamic sound pressure was 17 dynes/cm.^{2}. If we use the air pressure value of 2×10^{-4} dynes/cm.^{2} for human threshold of hearing (Sivian & White, 1938) then equation (1) becomes

$$x_A/x_w = (2 \times 10^{-4} \times 3700)/17$$
$$= 1/23 \text{ approx.}$$

Hence, the hydrodynamic displacements used were about twenty times the corresponding ones in air for human hearing threshold. Pumphrey (1950) has noted the transparency of marine animals to sound (since the densities on either side of the water/animal interface are very much nearer the same value than those between air and terrestrial animals); hence the displacements calculated above will reach the statocysts with little attenuation. The phase difference with which airborne sounds reach the mammalian cochlea, however, will be absent at the receptors of marine animals (except at wavelengths comparable with the inter-statocyst distance) and therefore the directional properties of hearing will be lost. Fraser & Purves (1960) have shown how this may be recovered in Cetacea by the acoustic insulation of each ear; but no comparable structures have been recognized in the octopus.

A special condition which applied to these experiments should be considered here. In any programme which involves the training of *Octopus* it is essential to maintain good sea-water circulation through the tanks in which the animals are kept (Boycott, 1954). At Naples high oxygenation of the refill liquid entering the tanks was ensured by means of a jet impinging from above on to the water surface. The animal was therefore living in a state of high background noise. This was 'heard' qualitatively by the hydrophone but no exact measurement was made of the sound pressures it produced. In order to counteract this, in some measure, the jet was diverted a minute or so before each trial was made (whether or not the sound was used) but this represents a very small fraction of the 24 hr. total circulation. Hence, the octopus may have achieved considerable adaptation to sound which did not necessarily disappear when the jet was diverted.

It may be that a more electrophysiological type of experiment—recording from the statocyst nerve during sound stimulation, for example—would be more rewarding. It would not, of course, answer the question of 'hearing' from the behavioural point of view, but it would at least establish whether a mechanism for such a faculty did exist.

SUMMARY

1. A description is given of apparatus suitable for transmitting hydrodynamic sound waves within a rectangular tank.

2. Two types of training experiment for investigating hearing in *Octopus* are described, one using simple training to attack, the other training to attack with discrimination.

3. These results of the experiments, when expressed in latencies of attack on food, gave little evidence that the octopus was able to hear.

4. Some factors which might have influenced the results are discussed together with suggestions for any future investigations.

I would like to record my appreciation of much technical advice from Mr R. Stubbs and the loan of the hydrophone from the National Institute of Oceanography. Acknowledgement is also due to the Royal Society and the Nuffield Foundation for part of the expenses involved in this work and also to Dr P. Dohrn of the Stazione Zoologica at Naples for laboratory facilities there.

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THE LANDING RESPONSES OF INSECTS

I. THE LANDING RESPONSE OF THE FLY, *LUCILIA SERICATA*, AND OTHER CALLIPHORINAE

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Although insect flight, and the stimuli which induce it, have been studied extensively, the way in which insects land has received little attention. The majority of insects fly with the pro- and meso- and in some cases the metathoracic legs folded up beneath the body and the legs must be lowered before the insect makes contact with the landing surface. This paper describes a detailed examination of the movements of the legs of the fly *Lucilia sericata* and other insects and an attempt to determine the stimuli which result in *Lucilia* lowering its legs on approaching a suitable landing surface. There are several possible stimuli which may cause the legs to be lowered; the object which the fly is approaching will appear to increase in size and this stimulus will of necessity be accompanied by a change in the light intensity falling on certain of the ommatidia. If the insect has a reasonable field of binocular vision, the lowering of the legs may be based upon an appreciation of its distance from the landing surface, together with its rate of approach to that surface. Alternatively, it may always lower the legs when the image of the approaching object falls simultaneously on corresponding ommatidia of the two eyes.

Hollick (1941) has shown that the stimulus of an air current on the antennae causes the legs of the fly *Muscina stabulans* to be raised up and held in the flight position. It is possible, therefore, that when an insect slows down to land the consequent reduction of air pressure on the antennae may cause the legs to be lowered.

In these investigations the stimuli which initially attract the fly to the landing surface were not considered.

MATERIALS AND METHODS

It was necessary to restrict the movements of the fly in such a way that, while the experimental conditions could be rigidly controlled, the results would, as far as possible, be applicable to the freely flying insect. In preliminary experiments flies were given varying degrees of freedom of movement ranging from suspension from a very light-weight pendulum allowing movement through 180° in the vertical plane in a fore and aft direction, to tethering with a 2 ft. length of nylon thread giving freedom of movement in all planes within a sphere of radius 2 ft. For the majority of experiments, however, it was found most convenient to have a stationary insect flying in an air stream and to move the landing surface towards it through known distances at controlled speeds. The responses of rigidly mounted flies, and those more freely suspended, were filmed and compared and are described later. The conditions under which the rigidly mounted fly responds appear to resemble closely those under which the freely flying insect responds.

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Specimens of *Lucilia sericata* were mounted after the method used by Hollick (1941). He determined the relationship of the flight system, i.e. the living fly and the surrounding air, under natural conditions in free flight and under experimental conditions with the insect held stationary. He found that when the living insect was held stationary with the body axis inclined at 10–29° to the horizontal and exposed to a stream of air whose speed ranged between 1·6 and 2·3 m./sec. it closely resembled in essentials the system in free flight. The actual flight system of the insect, therefore, was not interfered with in any significant way by mounting it in this manner, since it is likely that the conclusions reached for *Muscina stabulans* apply in a large measure to the closely related fly *Lucilia sericata*.

Flies which had emerged between 7 and 17 days previously were lightly anaesthetized and attached by the thorax to a fine piece of wire. The head was attached to the thorax by a narrow bridge of wax preventing head movements (see Fig. 3*a*). The wings were then blown forward until the insect started to fly, when it was left for ½ hr. to recover from the anaesthetic. It was then tested to ensure that recovery was complete. The criteria used were: (*a*) cessation of flight when the legs were brought into contact with the substratum; initiation if they were removed. (*b*) Initiation of flight if the abdomen or other parts of the body were touched. (*c*) Retraction of the legs if the antennae were stimulated with an air current.

It was very much easier to give a controlled stimulus, and to establish the angular relationship between the fly and the landing surface when the legs were lowered, if the fly was stationary and the landing surface itself moved. An apparatus was therefore constructed to enable suitable surfaces to be projected towards the fly through a measurable distance at a measurable uniform speed. Circular black disks were normally used of diameter ranging from 1·5 to 15 cm.; they were screwed to the end of a circular metal rod which was moved forward by the friction between it and a rotating pulley belt on to which it could be lowered. The speed of the pulley belt was controlled by a variable gear box, and the forward speed of the rod could be calculated from the speed of rotation of the pulley, since it was ensured that there was no slip between the two. The disks could be projected at any desired speed up to 150 cm./sec., the periods of acceleration and deceleration at the beginning and end of the run being very short.

The end of the rod bearing the disk projected into a white box 3 × 3 × 2 ft. (Fig. 1), provided with an observation panel. The carrier holding the fly and the jet of air in which it flew could be clamped in accurate alignment with the centre of the disk and the line of travel of the rod, the fly always being mounted in line with the centre of the disk unless otherwise stated. The disk started its travel flush with the wall of the box, the distance travelled being controlled by an adjustable rubber stop. This arrangement enabled objects of any required size to be projected at a known speed through a known distance towards the fly. It was possible to exchange the positions of the disk and fly if necessary and to fasten the fly to the rod so that it could be projected towards a stationary landing surface.

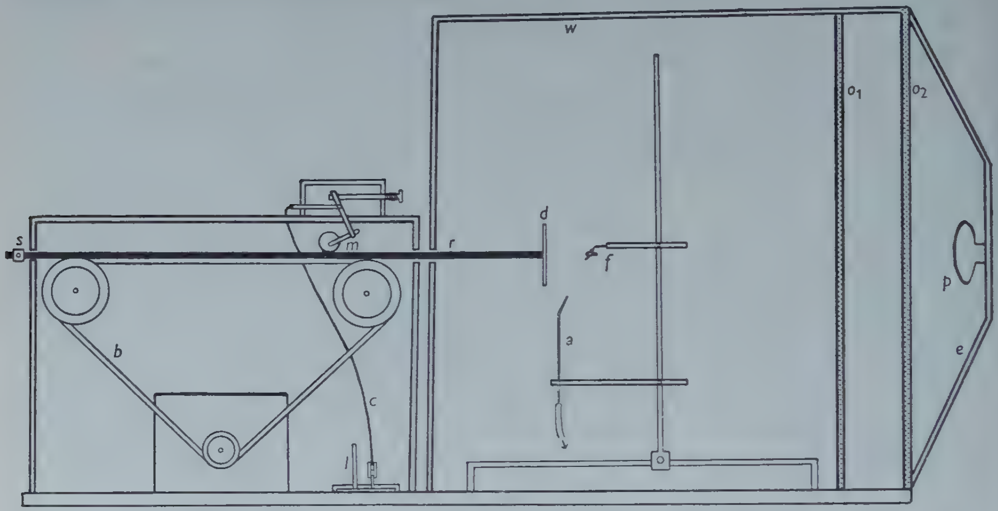

Fig. 1. Section through the apparatus used to move a landing surface towards the flying insect. *a*, Jet of air from the compressed air supply (arrow); *b*, pulley belt; *c*, Bowden cable; *d*, black disk screwed to the end of the rod; *e*, reflector; *f*, fly on adjustable support; *l*, lever, movement of this lever causes the wheel *m*, to be lowered on to the rod, pressing it firmly against the pulley belt so that it starts to move forward without slip: o_1 and o_2, sheets of opal glass; *p*, lamp; *r*, rod; *s*, stop; *w*, white-walled box.

The inside of the box and all the supports were painted a matt white to present an unbroken white surface providing the maximum contrast to the black or grey disk used. In some experiments the inner surface of the box and all supports were covered with a matt black surface to contrast with white and light grey disks. Illumination was by reflected light from a 24 V., 150 W. bulb, placed behind opal glass screens to eliminate shadows cast by the moving disk. Since the flicker fusion frequency of *Lucilia* is well above 50 cyc./sec. (unpublished observations), the lamp was run from two 12 V. car batteries. The light intensity in the box could be varied by means of a rheostat, and measured using an S.E.I. photometer reading directly in log. ft. lamberts.

A second apparatus was designed to stimulate the fly with a change of light intensity unaccompanied by movement in the visual field. A controlled increase or decrease of illumination was produced over a chosen area of the fly's visual field at a measurable speed, the change of intensity here being uniform over all the receptors involved. The fly was mounted in a matt black box facing a wall containing a circular opal glass disk, illuminated from outside by a lamp powered by batteries as before. The illumination of the screen was reduced by covering the lamp gradually with a light-tight metal canister and increased by withdrawing the canister from the lamp. The canister ran on wheels on two horizontal rails, and was screwed to one end of a rod moving on a pulley belt as described in the previous apparatus (Fig. 2A, B). To ensure that the light intensity of the screen would change uniformly over its surface, without the canister movement being visible, the lamp was mounted 1 ft. 10 in. away at a slight angle, and a second sheet of opal glass interposed near the lamp. The apparatus was then calibrated so that the position of the canister corresponding to a particular intensity on the opal disk was

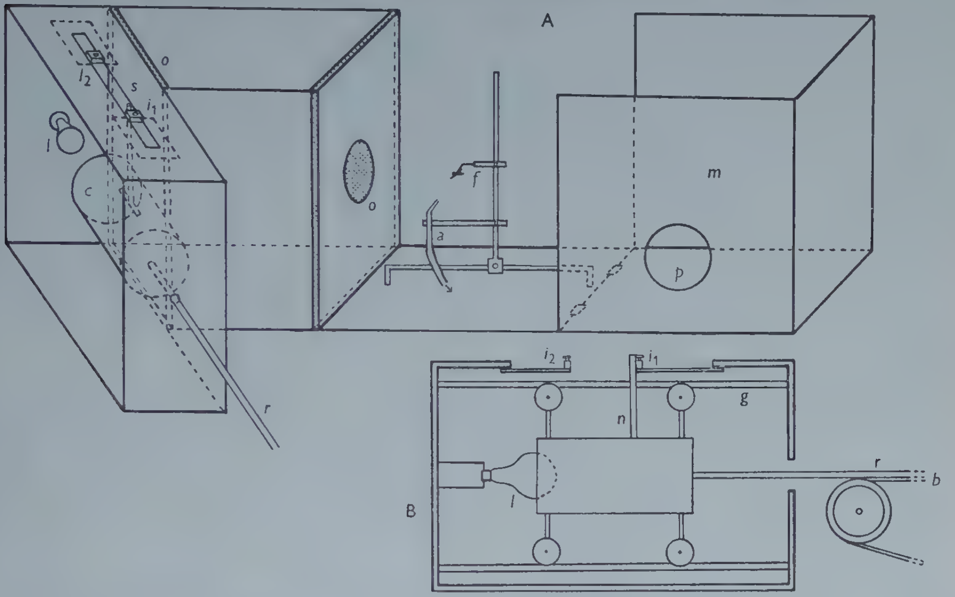

Fig. 2. (A) A side view of the apparatus used to produce a controlled change of light intensity over an opal glass disk by moving a metal canister over a lamp. Detail of the canister movement is omitted here and shown in Fig. 2 (B). *a*, jet of air; *b*, pulley belt; *c*, canister which moves on rails, *g*, to cover lamp *l*; *m*, metal box, opened to show the fly, *f*; *p*, observation panel used during experiments when the box is closed; *r*, rod screwed on to the end of the canister, and running on a pulley belt as in Fig. 1; *s*, slit in the roof of the box, the length of which can be adjusted by two moveable stops i_1 and i_2; *n*, metal rod projecting upwards from the canister enabling its movement to be regulated by the position of the stops. Fig. 2 (B) The canister is shown at the beginning of its run pulled back against the first stop, i_1. It runs forward along guide rails *g* on four small wheels.

indicated by a pointer moving over a horizontal scale. Thus by appropriate placing of stops any desired decrease or increase in light intensity of the disk could be produced. A lever was pressed which lowered the rod on to the pulley belt and the canister ran forward at a known speed through a known distance resulting in a uniform decrease of intensity over the disk. For an increase of intensity the direction in which the pulley belt moved was reversed. The rate of change in intensity was proportional to the speed of the canister. The fly was mounted as in the previously described apparatus, the centre of its head being in line with the centre of the circular opal glass screen. The area of the visual field stimulated by the change in light intensity could be controlled by changing the radius of the disk or the distance away of the fly.

Ciné films of the response

The response of rigidly mounted flies, stimulated by the approach of black disks of varying sizes, was filmed with a 16 mm. ciné camera (film speed 64 frames/sec.) so that leg movements might be studied in detail.

In the normal flight position the femur and tibia of the first and second pair of legs were folded up together at the side of the body with the tarsi extending forward. The third pair of legs streamed behind in the air current set up by the wings (Fig. 3*a*). When the disk was 3 or 4 in. away from the fly the tarsi of the first and second pair of legs began to be lowered (Fig. 3*b*). As the disk continued to approach the tibia and femur of the first two pairs of legs were extended forward; the second were unfolded and extended downward and slightly forward (Fig. 3*c*). When the disk was very near, the first and second pair of legs were completely unfolded and extended forward and the third pair were bent forward (Fig. 3*d*). As soon as the first pair of legs made contact with the disk the wings stopped beating and the body was bent round so that the first, the second and then the third pair of legs came in contact with the surface (Fig. 3*e*). Whenever the stimulus was adequate to elicit a response the movement of the legs followed this pattern exactly.

The camera speed was not fast enough to allow a detailed analysis of the wing movements. However, it was possible to detect, though not to measure accurately, a reduction in the rate of the wing beat as the insect began to lower its legs. This was accompanied by a change in the inclination of the stroke plane from the habitual 30–50° to the horizontal axis of the body to almost 90°, possibly a reaction which, if it took place in free flight, would tend to prevent the fly from stalling as it slowed down. This change in the rate and plane of the wing beat did not occur until the legs began to be lowered. The reactions of the legs and wings were exactly the same when rigidly mounted flies were moved towards stationary surfaces, and when flies tethered to 2 ft. of nylon thread were allowed to land on a stationary surface, or, while remaining fairly still themselves, had a landing surface moved towards them. Although it was not possible to film freely flying insects landing, the behaviour of free, hovering specimens of *Syrphus balteatus* on the slow approach of a suitable landing surface can be observed with the naked eye. The legs are lowered in a similar manner to *Lucilia* and, if observed in a strong beam of sunlight, the stroke plane can be seen to alter also. The uniformity of response of both legs and wings under these varied conditions suggest that this is the normal landing response of these insects, and that it is not modified in any way by moving the landing surface towards a stationary fly. However, the insect is normally free to vary its speed of approach to the landing surface, while under the experimental conditions the landing surface approaches at a constant speed. In all the filmed records the rate of wing beat appeared to remain fairly constant until the legs had begun to be lowered, when there was a marked slowing in the rate in spite of the fact that the stream of air in which the insect was flying remained constant. It seems likely therefore that the fly approaches surfaces at a fairly constant rate until it is quite near the landing surface; it then lowers the legs and slows down almost simultaneously. The timing of these two reactions suggests that the lowering of the legs is not the result of a sudden reduction in pressure on the antennae as the insect slows down or of marked changes in the apparent rate of approach of the insect to the object. It seems very probable that the behaviour of the insect under the experimental conditions closely resembles that of the freely flying insect.

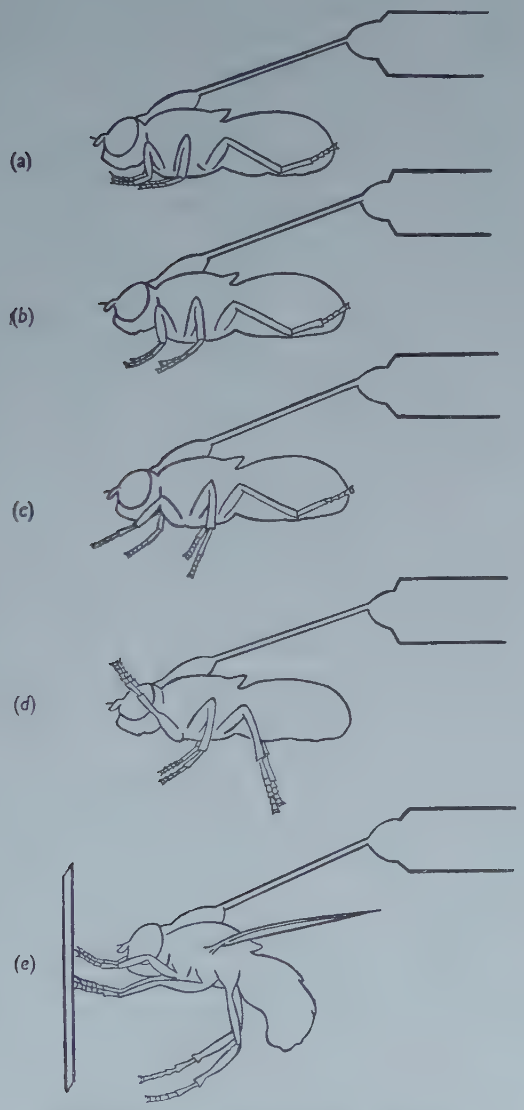

Fig. 3. (*a*) Shows the fly in the normal flight position with the 1st and 2nd pairs of legs folded up beneath the body and the 3rd pair streaming behind in the air current. (*b*–*d*) Four stages in the landing response. (*b*) The tarsi of the 1st and 2nd pairs of legs are lowered. (*c*) The tibia and femur of the 1st and 2nd pairs of legs are unfolded. (*d*) The 1st pair of legs are completely extended and stretched forward in front of the head. The 2nd pair begin to be extended forward. The 3rd pair are lowered. (*e*) Shows the fly landing on a vertical surface. As soon as the 1st and 2nd pairs of legs make contact with the surface the wings stop beating and the body is bent round so that the 3rd pair of legs make contact with the surface.

Stimuli which were inadequate to produce the full response were sometimes able to produce a partial unfolding of the legs, which would afterwards be returned to the normal flight position. Such partial unfoldings ranged from a very slight movement of the tibia of the first and second pairs of legs to a nearly complete unfolding of the legs, according to the strength of the stimulus. It was easy to see with the naked eye what stage in the unfolding of the legs was reached and so to assess the effectiveness of the stimuli. The unfolding of the legs was arbitrarily divided into three stages, corresponding to the three stages illustrated in Fig. 3*b–d*, to provide a convenient way of recording the degree of response.

The films also revealed that as the stimulus strength was reduced the fly's response was delayed until nearer the end of the disk's run. Finally, a little before the stimulus strength was so reduced that incomplete responses began to appear, the flies were responding just before the disk stopped moving. The experiments described in this paper are mainly concerned with finding the threshold stimulus for a full response. The experimental conditions are such, therefore, that if a fly responds it is always responding just before the disk stops moving, i.e. during the last 1–2 mm. of its travel. It can therefore be assumed, without appreciable error, that the distance between the fly and the disk when it has stopped moving is the same as the distance between the disk and the fly when it responded, a distance which it would otherwise be difficult to measure.

The sense organs mediating the response

It was possible that the fly's legs were lowered in response to a visual stimulus mediated by the compound eyes or the ocelli. Alternatively, as the insect slowed, a reduction in the pressure of the air current impinging upon the antennae might have caused the legs to drop. There also remained the possibility that under the experimental conditions air currents set up by the moving disk might stimulate the tactile hairs on the head and body of the fly.

The response of intact flies was compared with that of flies whose compound eyes or ocelli or both had been covered with an opaque black varnish and with flies whose antennae had been removed or whose heads had been enclosed in transparent celluloid capsules. In each case twenty flies were in turn stimulated ten times with a black disk 10 cm. in diameter approaching against a white background at 50 cm./sec., with and without a glass screen between the disk and the fly. The positions of the disk and the fly were then reversed and the fly was projected towards the disk. The flies were also stimulated by rapidly increasing or decreasing the pressure of the jet of air in which they were flying. The results for all flies are summarized in Table 1.

It is clear that the response is not affected by air currents set up by the approaching disk, since it is given in exactly the same manner when the fly is shielded from such currents by a glass plate. The legs appear to be lowered in response to a visual stimulus mediated by the compound eyes alone. The presence of the intact antennae is not necessary for the response to be given in a normal manner. Increasing the pressure on the antennae as the disk approaches (by opening the tap

Table 1

imulus (10 stimuli iven to each fly)	Insect intact	Compound eyes covered with opaque black varnish	Ocelli covered	Compound eyes and ocelli covered	Antennae cut off at base with razor blade	Head enclosed in celloidin capsule (after Hollick, 1941)	Blind with antennae removed
ck disk approach- g against white ackground	200 full responses	No response	200 full responses	No response	200 full responses	200 full responses	No response
eet of glass etween approach- g disk and fly	200 full responses	No response	200 full responses	No response	200 full responses	200 full responses	No response
projected to- ards stationary isk	200 full responses	No response	200 full responses	No response	200 full responses	200 full responses	No response
pressure on ntennae increased disk approaches	200 full responses	No response	200 full responses	No response	200 full responses	200 full responses	No response
dden reduction in ressure of current air impinging on ntennae	22 full responses, 33 stage 1–3 responses	27 full responses, 16 stage 1–3 responses	19 full responses, 26 stage 1–3 reponses	15 full responses, 28 stage 1–3 responses,	No response	No response	No response

controlling the compressed air supply), to compensate for any reduction in pressure due to alteration in the wing beat rate, does not prevent the response from being given. If the reduction in pressure was great enough some flies would lower their legs in response to this stimulus alone. However, the reduction in pressure necessary was much greater than would normally be caused by the insect slowing in flight. The flies rapidly became adapted to this stimulus and after one or two full responses, followed by some incomplete ones, the legs were not lowered.

The range of visual stimuli which elicit the response

Tests were made to determine which visual stimuli would elicit the landing response. Twenty flies were each stimulated in turn with objects moved towards and away from them, objects appearing to change in size without moving, and changes in the light intensity of the surroundings. In addition they were themselves moved towards and away from objects. In order to relate, as far as possible, the behaviour of the rigidly fixed specimens to that of the freely flying insects, the tests were repeated wherever possible with flies tethered to 2 ft. of nylon thread giving them considerable freedom of movement. Under these conditions the insects either fly around within the radius permitted by the thread or else, although flying, remain more or less in the same position.

Disks 9 cm. in diameter were projected a distance of 25 cm. towards the flies at a speed of 40 cm./sec. finishing their run 2·5 cm. away from the flies. The same speed and distances were used when the flies were projected towards the disk. In order to produce the effect of a change in size of the object, without a change in its distance from the fly, a diaphragm 6 cm. in diameter, inserted into a sheet of cardboard, was placed 4 cm. away from the flies and opened and closed in 1 sec.

The light intensity was varied by 1·0 log ft. lamberts in 1 sec. Each fly was given ten tests and its response noted. The results are summarized in Table 2.

The tests showed that it was not essential for the fly to be stimulated by a moving object, or by an object apparently increasing in size in order to elicit the landing response. Under certain circumstances, for example, a white disk retreating against a black background or a black diaphragm closing, the fly will respond to an object appearing to decrease in size. In addition a general decrease in the light intensity of the surroundings, involving no movement in the visual field, proved a very effective visual stimulus. The lowering of the legs does not, therefore, depend upon there being a landing surface at a given distance from the fly.

Much the most effective visual stimulus was one which produced a decrease of intensity in the visual field, for example, a black disk approaching against a white

Table 2

Stimulus	Contrast	Suspension	Response
Disk moving towards fly	Black disk against white background	Condition A. Fly rigidly attached to wire support as in Fig. 3*a*	100% response
		Condition B. Fly attached by thorax to 2 ft. of nylon thread	If fly moving as well as disk it avoids disk. If fly stationary landing response is given
	White disk against black background	Condition A	Less than 50% response
		Condition B	If fly moving as well as disk it avoids disk. If fly stationary occasionally gives response
Fly moving towards disk	Black disk against white background	Condition A	100% response
		Condition B	100% response
	White disk against black background	Condition A	Less than 50% response
		Condition B	Less than 50% response
Disk moving away from fly	Black disk against white background	Condition A	No response
		Condition B	No response
	White disk against black background	Condition A	Between 75–100% response
		Condition B	If fly moving no response, Between 75–100% response if stationary
Diaphragm opening	White diaphragm opening to reveal black aperture	Condition A	100% response
	Black diaphragm opening to reveal white aperture	Condition A	Less than 50% response
Diaphragm closing	White diaphragm closing over black aperture	Condition A	No response
	Black diaphragm closing over white aperture	Condition A	75–100% response
Increase of intensity	—	Condition A	No response
Decrease of intensity	—	Condition A	100% response

background, a white disk retreating against a black background, a white diaphragm opening and revealing a black surface, a black diaphragm closing over a white surface and a general decrease in the light intensity of the surroundings. A white disk approaching against a black background and a black diaphragm opening to reveal a white surface occasionally evoked a response, but the flies never responded to the withdrawal of a black disk against a white background, or to a general increase in the light intensity of the surroundings. Stimuli causing an increase of intensity over part of the visual field appear to be effective only when accompanied by an increase in the size of the stimulating object. It appears that this latter factor may, on occasion, play a part in eliciting the response though it is certainly not essential.

Controlled stimulation of insects attached to nylon thread was difficult. Some insects flew around within the limits imposed by the tethering thread and avoided the moving disk. Others, while flying, remained in more or less the same position and gave the landing response when the disk had approached to within 1–3 cm. of them. Other insects supported in this way were moved towards stationary disks and these too gave the landing response. According to Hertz (1934) bees are able to discriminate between moving objects and the apparent movement of objects due to their own movement. *Lucilia* also appears to be able to do this in some way, the moving fly distinguishing between a comparatively large moving object which it avoids and the same object stationary on which it prepares to land.

Flies do not discriminate, at least in their behaviour, between approaching a stationary disk and, while stationary, being approached by a disk. The landing response given in both cases is identical in all respects. It seems valid, therefore, to assume that the behaviour of the stationary fly stimulated by an approaching disk resembles closely that of the freely flying insect approaching a stationary surface.

Since in these preliminary tests the most effective stimuli were the approach of a dark disk against a light background and a general decrease in the light intensity of the surroundings, these stimuli and the responses given to them were analysed in greater detail.

Flies stimulated by an approaching disk

As the disk approaches it will appear to increase in size and certain ommatidia will be stimulated by a change of light intensity, the number depending (amongst other factors) upon the size of the disk, its distance from the fly and the distance it travels. The rate at which these ommatidia are stimulated depends upon the speed of approach of the disk. Another possible stimulating factor is the rate of change of angular size of the disk which varies continuously as it approaches the fly. In addition the contrast between the disk and its background may affect the response given. The effect on the landing response of varying each of these factors in turn was examined.

The simplest estimate of the area of the fly's visual field traversed by the edge of a disk as it approached was obtained by calculating the increase in the solid angle subtended at the eye by the disk as it moved forward. Owing to the rather complex geometry of the fly's eye it would be difficult to calculate accurately the exact number of ommatidia stimulated by the disk, even if the variation in ommatidial angle were known in detail over the whole eye. The ommatidial angle was measured by cutting sections in the horizontal and vertical planes of the eye. It remained fairly constant over the central part of the eye at 2° in both the horizontal and vertical planes, increasing to 5° at the outer edge of the eye. As the basis of an approximate calculation it has been assumed that the eye is a hemisphere and the ommatidial angle constant. In such an ideal case the number of ommatidia stimulated would be proportional to the change in solid angle ($\cos\theta_1 - \cos\theta_2$) where $2\theta_1$ and $2\theta_2$ are the angles subtended by the disk at the beginning and end of its run. Angle θ, the change in the angle subtended by the disk when it moves forward is thus ($2\theta_2 - 2\theta_1$). In the range of angles used in these experiments (20–100°) θ is approximately proportional to the change in solid angle, at least to within the limits of experimental error. It has therefore been used for convenience as an approximate estimate of the number of ommatidia stimulated. Accurate proportionality would not be expected for large values of θ where the ommatidial angle is not constant, or for small values of θ where the proportionality to change in solid angle breaks down. In addition, Burtt & Catton (1954) have shown that although the inclination of the ommatidial axes in the locust eye was 2·4° in the horizontal and 1° in the vertical meridian of the eye, the actual visual field of one ommatidium was approximately 20°. They suggest that this overlapping of ommatidial fields is general amongst insect eyes and if this is so in the case of *Lucilia* the number of ommatidia stimulated for a given value of θ will be greater than that expected. The effect of an increase in the field of vision of the ommatidia would be to restrict slightly the upper limit of the range over which the calculation applies. Over the greater part of the experimental range, however, θ gives a fair measure of the number of ommatidia stimulated by the disk.

The rate of change of angular size of the approaching disk depends upon its radius, its speed of approach and its distance from the fly and can be calculated as follows.

Let p be the radius of the disk in cm.; v be the disk velocity in cm./sec.; x be the distance of the disk from the fly and α be the angle subtended by a radius of the disk (in radians).

$$\frac{d\alpha}{dt} = \frac{d\alpha}{d(\cot\alpha)}\,\frac{d(\cot\alpha)}{dx}\,\frac{dx}{dt}$$

$$= -\frac{p^2}{x^2+p^2}\,\frac{1}{p}\,v$$

$$= \frac{v}{p}\,\frac{p^2}{x^2+p^2}\ \text{radians/sec.}$$

$$= 57\,\frac{v}{p}\,\frac{p^2}{x^2+p^2}$$

$$= \frac{57vp}{x^2+p^2}\ \text{degrees/sec.}$$

Since the disk diameter, the distance x and the disk velocity are all known, the rate of change of angular size at the moment when the fly responds can be calculated.

The fly was mounted facing a disk, 9 cm. in diameter, which travelled forward 25 cm. and stopped 10 cm. away from the fly. The speed of the disk was varied until, by trial and error, a point was found at which the fly gave a full response every time it was stimulated. The fly was then stimulated ten times at $\frac{1}{2}$ min. intervals at this speed and the degree of unfolding of the legs, if any, resulting from each stimulus was recorded. The speed was then reduced by a known amount, and a further ten stimuli given. This was repeated until the fly ceased to respond. The speed was then increased by the same steps and the tests repeated. After an interval of $\frac{1}{4}$ hr. the two sets of tests were repeated and after a second interval the fly was stimulated for the third time. The average percentage of full responses given at each speed was then determined and plotted against the rate of approach of the disk, measured in cm./sec. Similar graphs could also be plotted for each of the other stages in the unfolding of the legs if desired.

This method is only valid if the variation in the percentage response with the strength of the stimulus remains constant so that reproducible results are obtained. In order to test this, and to determine whether the fly became adapted to or fatigued by the stimulus, an experiment similar to that described above was performed, except that only two series of ten tests were given. The stimulus required to obtain 50% response was read off from the graph and the fly was stimulated 100 times at this stimulus strength (in this case 31 cm./sec.) at $\frac{1}{2}$ min. intervals, the type of response given being recorded as usual. This was repeated for a further two groups of 100 stimuli. Finally, the initial experiment in which the stimulus was varied was repeated. The two graphs plotted from the two sets of results obtained from one fly at an interval of three hours were identical (see Fig. 4). The percentage of full responses given in each of the groups of 100 tests was compared with the percentage response read off from the two graphs at the stimulus value used. The five sets of results obtained, 50, 52, 47, 51, and 50%, agree to within 5%. The results of this experiment justified the use of the method previously described of estimating the average percentage response given to a particular stimulus from a series of ten tests, repeated several times, and showed that the results were reproducible over a period of several hours. In a further test flies were stimulated at a level just above the threshold for full responses at 5 sec. intervals to determine how long they would respond. As long as they continued to fly, which they did for periods of from 1–5 hr. and in one case 8 hr., they gave the full response to each stimulus. If fed with glucose solution upon cessation of flight they would very often begin to fly again and would continue to give the full response. Flies thus showed no signs of becoming adapted to or fatigued by this stimulus.

Stimulation by disks (a) of different diameters, and (b) travelling different distances

Ten flies were each stimulated by eleven black disks whose diameters ranged from 2 to 12 cm. The disks travelled 30 cm. towards the flies against a white background at a speed of 30 cm./sec., stopping 10 cm. away from them. Fig. 5 shows that the average percentage of full responses given by the ten flies was directly proportional to the disk diameter.

Ten flies were stimulated in turn by a disk 9 cm. in diameter starting 35 cm. away from them and approaching at 40 cm./sec. The distance travelled by the disk was varied between 15 and 25 cm. The average percentage of full responses given by the ten flies was directly proportional to the distance travelled by the disk, a greater response being obtained the nearer the disk approached (Fig. 6).

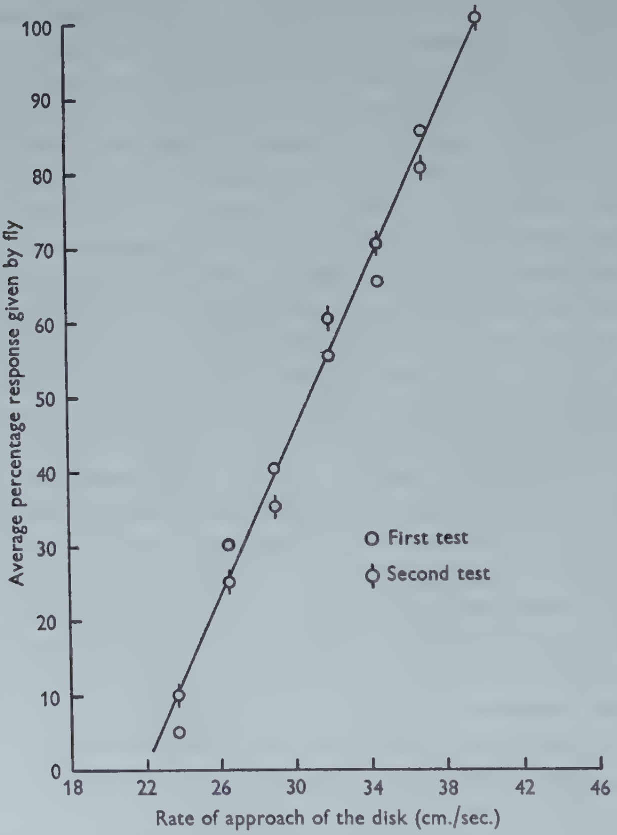

Fig. 4. Relationship between the average percentage response given by one fly and the rate of approach of the disk.

It was possible to replot the results of the two previous experiments in terms of the increase in the angle subtended at the eye by the disk (θ) and the rate of change of angular size of the disk at the point at which the flies responded (see Figs. 7*a*, *b* and 8*a*, *b*). The response given by the flies is seen to be directly proportional to the increase in the angle subtended at the eye by the disk. It is not, however, directly related to the rate of change of angular size of the object.

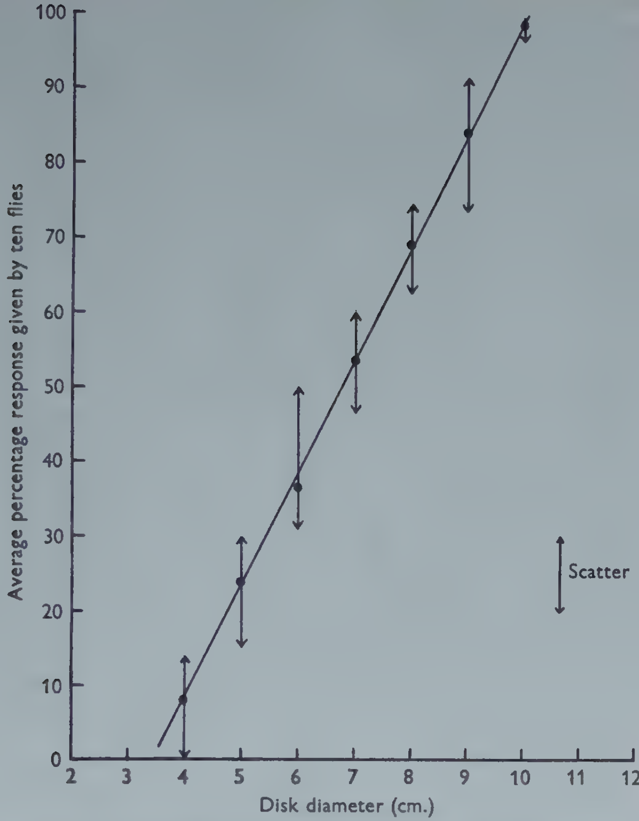

Fig. 5. Relationship between the average percentage response given by ten flies and the diameter of the approaching disk.

The threshold stimulus required for 100% response

Eighteen flies were each tested with thirteen disks having diameters ranging from 3 to 15 cm. and travelling 30 cm. at 50 cm./sec. The distance between the fly and each disk at the end of its run (hereafter known as distance x) was varied and the percentage response given was plotted against the value of distance x. The value of distance x necessary for 100% response with each disk used could then be read off from the graphs. The stimulus necessary for 100% response in each of the tests was tabulated in terms of the disk diameter and distance x required, the value of θ and of the rate of change of angular size which this represents (see Table 3).

The results show that the flies do not always respond at a fixed distance from the approaching object, but at such a distance that the increase in the angle subtended at the eye is approximately the same whatever the size of the approaching object. Since θ is approximately proportional to the number of ommatidia stimulated it appears that to obtain a given response a given number of ommatidia must be stimulated by a change in light intensity as the disk passes across their visual field.

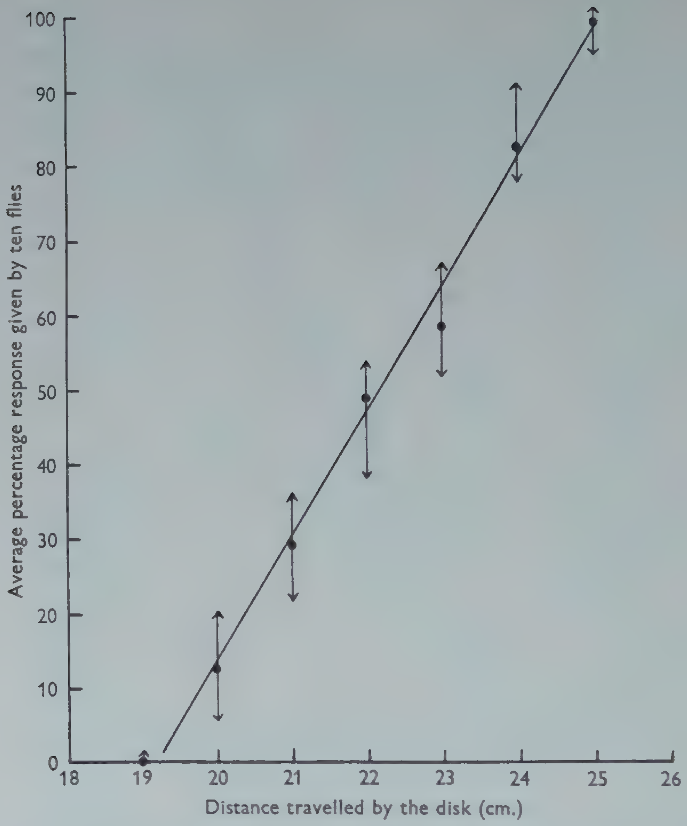

Fig. 6. Relationship between the average percentage response given by ten flies and the distance through which the disk travels.

Variation in the rate at which the disks approached

Ten flies were stimulated in turn with a black disk, 9 cm. in diameter, travelling 25 cm. at speeds varying between 18·5 and 40 cm./sec. The percentage of full responses given by each fly was plotted against the rate of approach of the disk (cm./sec.). In each case the percentage response given was directly proportional to the rate of approach of the disk, as previously shown in Fig. 4.

The effect of varying the contrast between the landing surface and the background

The visual differentiation of an object depends upon the contrast presented when the object and its background differ in brightness or in colour or in both these characteristics. In order to present a simple stimulus situation the contrast in these experiments was confined to differences in physical brightness only. Grey disks of a wide range of shades were used. They were presented first against a white background and then against a black background. The contrast between the disk

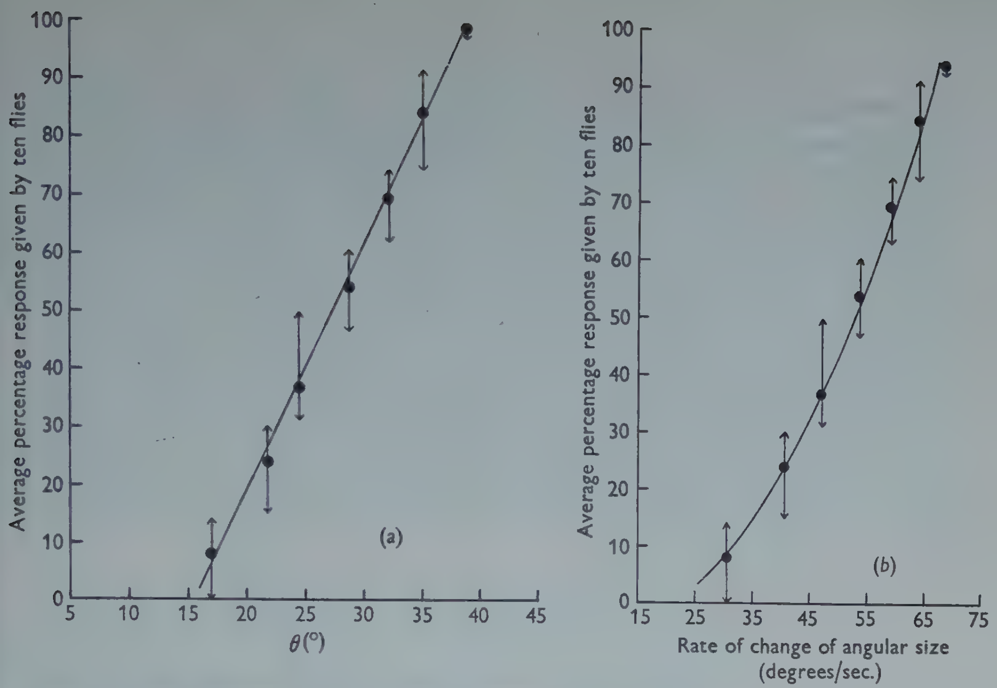

Fig. 7. (*a*) Results shown in Fig. 6 replotted to show the relationship between the average percentage response given by ten flies and the difference between the angles subtended at the eye by the disk at the beginning and at the end of its run (θ). (*b*) Results shown in Fig. 6 replotted to show the relationship between the average percentage response given by ten flies and the rate of change of angular size of the disk at the moment the flies respond.

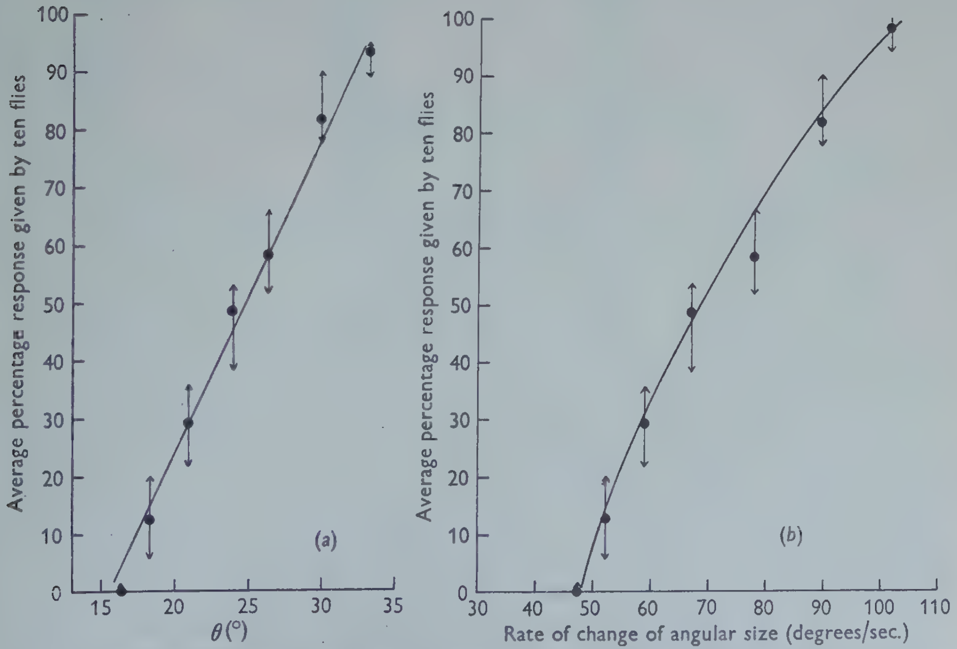

Fig. 8. (*a*) Relationship between the average percentage response given by ten flies and θ (the increase in the angle subtended at the eye by the disk as it moves forward). θ was varied by varying the distance travelled by the disk. (*b*) Relationship between the average percentage response given by ten flies and the rate of change of angular size of the disk at the moment when the flies responded.

and the background was measured in terms of the relative brightness difference $(I_1-I_2)/I_1$, where I_1 is the brightness of the background and I_2 is the brightness of the disk measured in log ft. lamberts, since brightness contrast depends upon relative rather than absolute brightness differences (Weston, 1945). The brightness of a surface is a function of the illumination it receives and of its reflexion factor. At the start of the disk's run it is flat against the wall of the box and at this stage the disk and the background receive practically identical illumination. The brightness contrast is, therefore, a measure of the difference between their reflexion factors. As the disk moves towards the fly it moves nearer the light source and its illumination increases according to the law of inverse squares. However, the forward movement is small compared with the distance from the light source and measurements of the light intensity of the disk at the beginning and end of its run showed that there was very little error involved if the average of these two intensities was taken as the brightness of the disk.

Table 3. *The threshold stimulus necessary to achieve* 100% *response*

Disk diameter (cm.)	Average distance between the disk and the fly at which the fly gave 100% response (cm.)	Average increase in the angle subtended at the eye by the disk (θ) at which the fly gave 100% response	The rate of change of angular size of the disk (degrees/sec.) at the moment when the fly responded
	Lucilia sericata		
3	5·36	26° 22′	138·00
4	6·94	26°	109·20
5	8·21	26° 40′	96·74
6	9·73	25° 50′	82·47
7	10·92	26° 14′	75·88
8	12·03	26° 20′	70·98
9	12·90	26° 30′	68·71
10	14·16	26° 12′	63·20
11	15·07	26° 8′	60·91
12	15·92	26° 49′	59·07
13	16·85	26° 17′	57·71
14	17·71	26° 38′	55·13
15	18·68	26° 53′	52·85
	Muscina stabulans		
1·5	3·21	23° 8′	196·8
3	6·36	21° 12′	100·2
5	9·02	22° 53′	81·34
7	12·00	22° 45′	63·84
9	14·47	24° 20′	55·86

Ten flies were stimulated in turn with fifteen disks, 9 cm. in diameter and of varying shades of grey, projected against a white background. The disks approached at a speed of 20 cm./sec. and the value of θ was 60°. The average percentage of full responses given by the flies was directly proportional to the relative brightness difference measured directly in log ft lamberts (see Fig. 9). The experiment was repeated with θ equal to 100, 80, 40 and 30°, although for the latter two values it was necessary to increase the rate of approach to 45 cm./sec. to obtain 100% response. A similar result was obtained in each case. The walls of the box were then covered with a matt black paper and the disks projected against a black background. The response obtained was not so consistent as when a white background was used, some flies responding very infrequently, while in no case was the response directly related to the relative brightness difference.

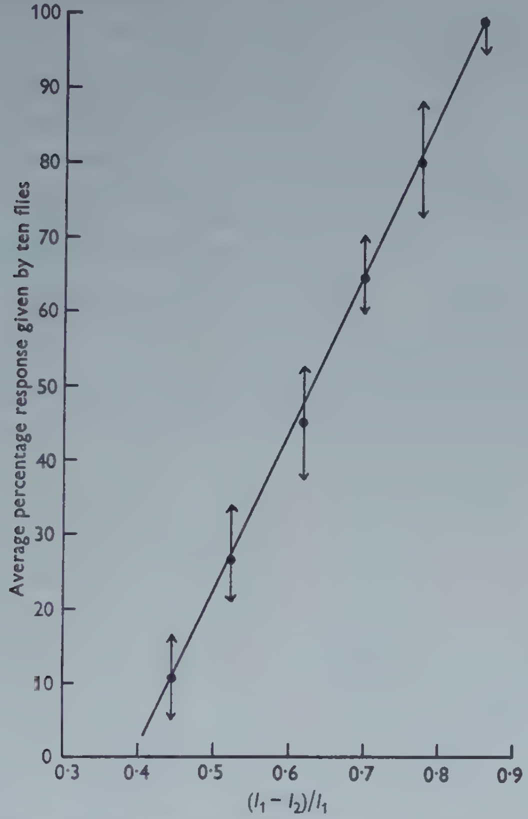

Fig. 9. Relationship between the average percentage response given by ten flies and the relative brightness decrease as a dark disk approached against a white background.

The relationship between θ, the rate of approach of the disk and the contrast

Since the percentage response given by the flies was directly proportional to the increase in the angle subtended at the eye by the disk (θ), the rate at which it approaches (V) and its contrast with the background $(I_1 - I_2)/I_1$, it seemed likely that there might be some relationship between these three factors.

The relationship between θ and V was first examined, keeping $(I_1 - I_2)/I_1$ constant, the speed necessary to achieve 100% response being determined for different values of θ. Ten flies were stimulated by a black disk, 9 cm. in diameter, with a background intensity of 1·5 log ft. lamberts. θ was first set at 20° and the rate at which the disk approached was varied, the percentage response given by each fly being plotted against the rate of approach of the disk. θ was increased by intervals of 10° and the experiment was repeated. The rate of approach of the disk necessary to produce 100% response for each value of θ was read off from the graphs. Fig. 10

shows that the log of these rates of approach plotted against the log of the values of θ yields a straight line of slope -1. The rate at which the disk must approach the fly in order to produce 100% response is therefore inversely proportional to the increase in the angle subtended at the eye by the disk. Results obtained from other experiments have been included in Fig. 10 for comparative purposes and show close agreement.

The relationship of θ, V, and $(I_1 - I_2)/I_1$ was examined using the results of previous experiments. The rate of approach necessary to obtain 100% response when each of the grey disks was used was known for a wide range of values of θ. Fig. 11 shows the relationship between the product θV and $(I_1 - I_2)/I_1$ in each case. θV is inversely proportion al to $(I_1 - I_2)/I_1$, i.e. $\theta V \frac{(I_1 - I_2)}{I_1}$ is a constant for a constant response. The response given by the fly is therefore directly proportional to the product of $\theta V \frac{(I_1 - I_2)}{I_1}$. These results suggest that the response given by the flies is directly proportional to the total decrease of intensity (the number of ommatidia stimulated × the relative brightness decrease) × the rate of decrease.

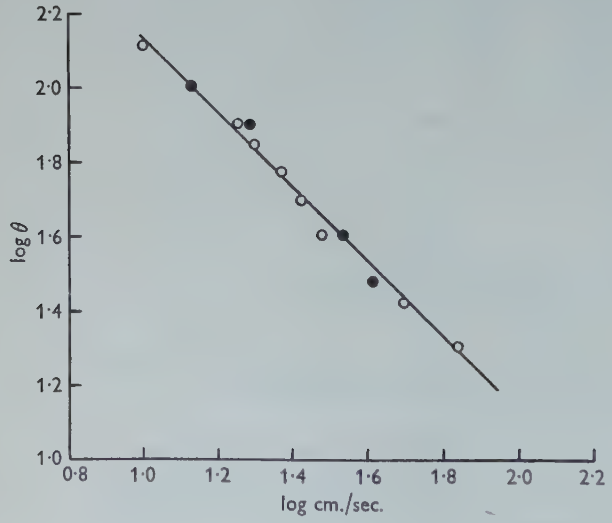

Fig. 10

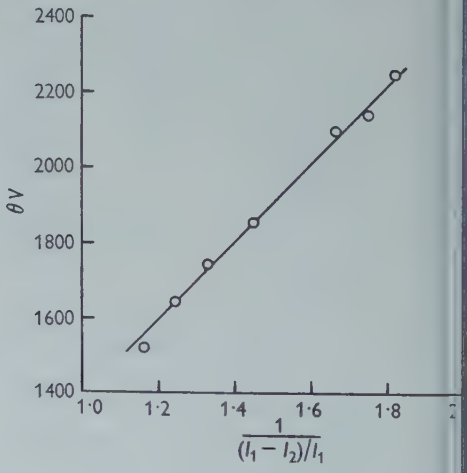

Fig. 11

Fig. 10. The relationship between θ and the rate at which the disk must approach the fly in order to produce 100% response. The filled circles represent results obtained from some of the previous experiments.

Fig. 11. The relationship between the product of θ V (V being the rate of approach to the disk in cm./sec.) and the relative brightness decrease $(I_1 - I_2)/I_1$ required for 100% response.

Stimulation by a simple change of light intensity

If the effective stimulus given by an approaching object is the decrease of intensity it produces, it should be possible to elicit the response by stimulating the flies with a decrease of light intensity which does not involve a moving object. The apparatus used for these experiments has been described above. The fly faced an opal glass disk the intensity of which could be decreased by a known amount at a known rate. An estimation of the total visual field of the two eyes affected was obtained by calculating the angle subtended at the eye by the opal glass disk in one plane. This angle is hereafter known as angle θ_2. In the range of values used it is approximately proportional to the number of ommatidia stimulated by the change in light intensity, assuming as before a hemispherical eye and a constant ommatidial angle.

The experiments performed with a moving disk were repeated with an increase and then a decrease of light intensity as the stimulus, using the same technique as before. θ_2, the relative brightness change $(I_3-I_4)/I_3$ measured in log ft. lamberts (where I_3 is the intensity at the beginning and I_4 the intensity at the end of the change), and the rate of the change (V_2) were varied in turn. No fly ever responded when the intensity of the opal disk was increased. A normal landing response was given when the intensity was decreased and figs. 12, 13 and 14 show that the average percentage of full responses given by the ten flies tested was directly proportional to θ_2, to the rate of the decrease measured in terms of the speed at which the canister moved over the light source, and to the relative brightness decrease $(I_3-I_4)/I_3$.

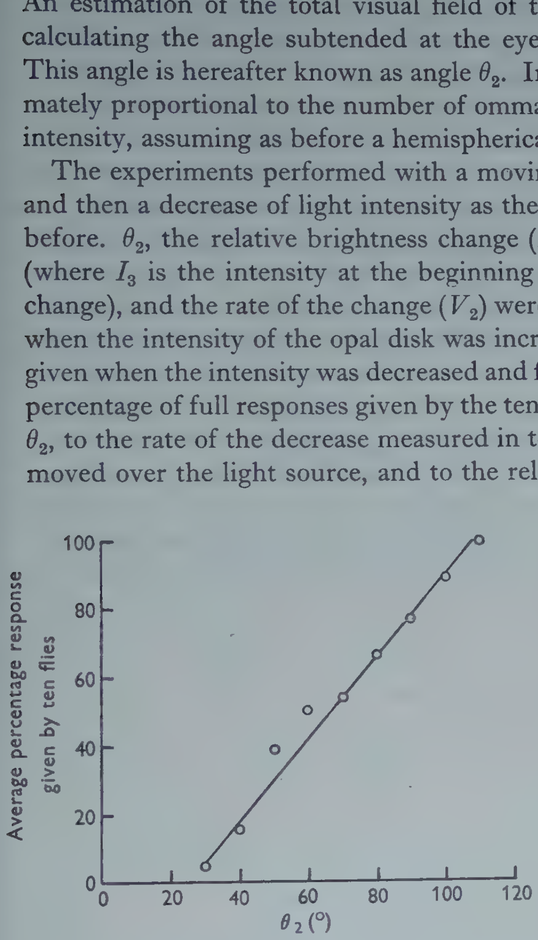

Fig. 12

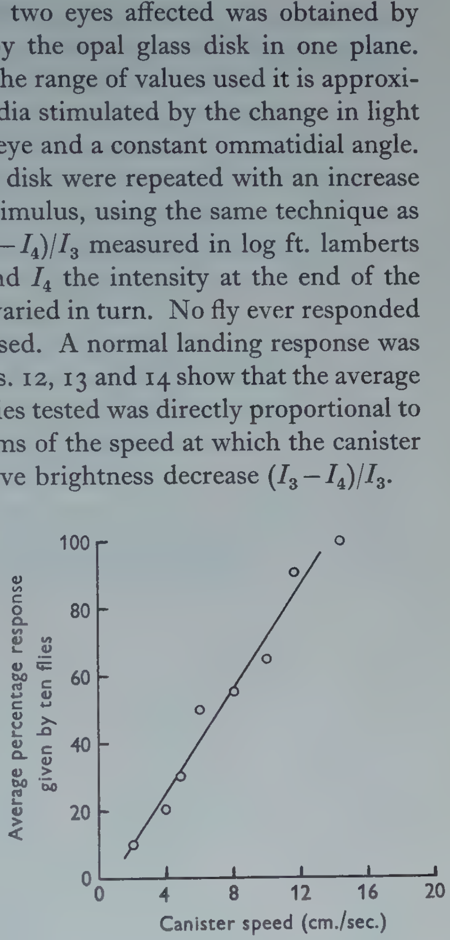

Fig. 13

Fig. 12. The relationship between the average percentage response given by ten flies and the angle subtended at the eye by the opal glass screen (θ_2).

Fig. 13. The relationship between the average percentage response given by ten flies and the rate of the decrease of intensity of the opal glass screen (V_2). (Measured in terms of the rate at which the canister was moved.)

The relationship between θ_2, V_2 and $(I_3-I_4)/I_3$ was also investigated. The rate of decrease of intensity necessary for 100% response was determined for values of θ_2 ranging from 30 to 100° for a given value of $(I_3-I_4)/I_3$. A slope of approximately -1 was obtained when the log of the rate of decrease was plotted against the log of θ_2 (see Fig. 15), indicating that the rate of the decrease of intensity required for 100% response was inversely proportional to θ_2. Similarly the rate of decrease necessary for 100% response was found for various values of $(I_3-I_4)/I_3$ when θ_2

was constant. The rate of decrease of intensity required for 100% response was inversely proportional to $(I_3 - I_4)/I_3$ (see Fig. 16). The percentage of full responses given by the flies is therefore directly proportional to the product of θ_2 (and hence to the number of ommatidia stimulated), the relative decrease of intensity and the rate of the decrease. A decrease of light intensity which in no way involves movement in the visual field produced a response identical with, and dependent upon, the product of the same three factors as the response given to a dark object approaching against a light background.

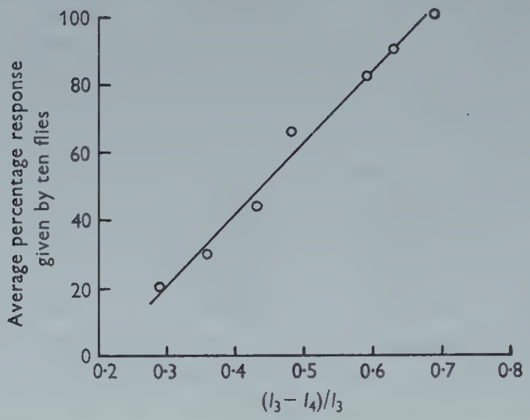

Fig. 14. Relationship between the average percentage response given by ten flies and the relative brightness decrease of the opal glass screen $(I_3 - I_4)/I_3$.

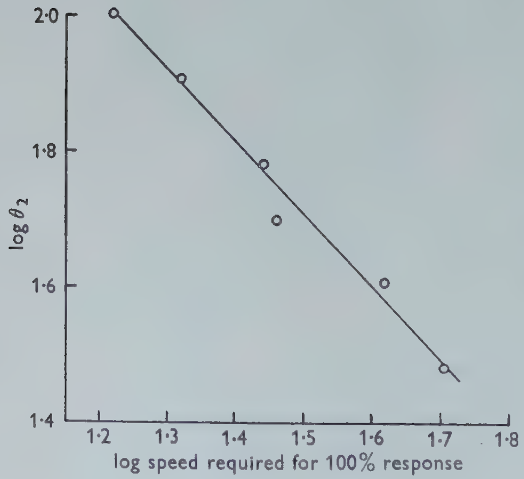

Fig. 15

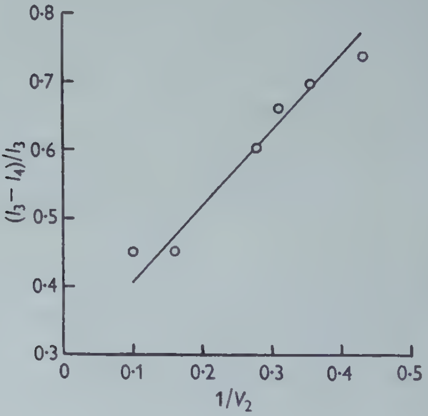

Fig. 16

Fig. 15. The relationship between θ_2 and the canister speed required for 100% response.

Fig. 16. The relationship between the relative brightness decrease $(I_3 - I_4)/I_3$ and the canister speed required for 100% response.

Occlusion of one eye

Nine flies were each stimulated by a black disk approaching against a white background. $(I_1-I_2)/I_1$ was kept constant at 0·86 throughout the experiment. The disk approached at 50 cm./sec. when stimulating the first three flies. The speed was reduced to 40 cm./sec. for the next three flies and to 30 cm./sec. for the last three flies used. θ was varied in each case and the percentage response obtained plotted against the value of θ. One compound eye and the ocelli of each fly were then completely covered with a quick-drying black varnish and the experiment was repeated. With one eye covered the visual field will be restricted, though not halved, because the visual fields of the two eyes overlap; but the actual number of ommatidia stimulated will, however, be reduced by half.

All the flies responded normally when one compound eye was covered; binocular vision is therefore not essential for the response. Table 4 compares the values of θ necessary for 100% response when the eyes are intact and when only one compound eye is functioning. The value of θ required is approximately doubled, confirming the implication of the previous results that the response is proportional to the product of $\theta V\,(I_1-I_2)/I_1$ at least over the range of values of θ, V and $(I_1-I_2)/I_1$ used in these experiments, values which are within the limits of those normally encountered by the fly.

Table 4

Speed (cm./sec.)	$(I_1-I_2)/I_1$	θ both eyes	θ one eye
50	0·86	27° 15′	56° 30′
		24°	47° 40′
		26° 39′	48°
40	—	32° 15′	60°
		34°	66° 30′
		30° 30′	63°
30	—	39° 40′	78° 49′
		43°	71° 30′
		41° 15′	79°

The landing responses of other flying insects

The leg movements during landing of many Diptera and representatives of other insect orders have been observed. The results are summarized in Table 5. Those insects which fold their legs beneath the body in some way during flight all lower at least the first and second pair of legs and extend them forward when approached by a suitable landing surface and most give a similar response to a decrease of light intensity. The response is very marked in those members of the Odonata which have been tested, all legs being extended forward to form a 'basket' with its open end directed forwards. It is possible that this reaction to an approaching object is utilized by the insect when catching prey on the wing.

DISCUSSION

The fly, *Lucilia sericata*, lowers its legs from their retracted flight position before landing in response to a visual stimulus to the compound eyes. Although by virtue of its overlapping frontal fields of vision the fly is presumably capable of some degree of distance estimation by a binocular method, the lowering of the legs is not based upon an estimation of the distance between the fly and the landing surface. The approach to a landing surface, at least as far as lowering the legs is concerned, and probably also with reduction in speed, is measured in terms of the product of: the

Table 5

Insect tested	Position of legs in flight	Response to approaching disk	Response to a decrease of light intensity
Orthoptera *Schistocerca gregaria*	Ist and 2nd pair of legs folded against the head and prothorax. 3rd pair folded along the side of the abdomen	1st and 2nd pair of legs unfolded and extended forward and lowered. Not given consistently	No response
Odonata *Aeschna juncea*	All three pairs of legs are folded up closely beneath the thorax	All the legs are lowered and extended forming a 'basket'. (Possibly this reaction is utilized when insects are caught on the wing)	Legs are lowered as with disk
Plecoptera sp.	Legs hang down beneath body	No response	No response
Ephemeroptera sp.	Legs hang down beneath body	No response	No response
Trichoptera sp.	Drawn up beneath the body	All legs lowered. 1st and 2nd pair extended forward	Legs lowered as with disk
Lepidoptera *Pieris brassica*	Tibia and femur folded together with tarsi extending backwards along the side of the body	1st pair of legs extended forward. 2nd and 3rd lowered slightly. Antennae also extended forward	Leg movements as with disk. Night flying moths show a marked change of flight posture. The antennae are extended. The rate of wing beat is increased
Hymenoptera *Bombus terrestris*	Legs folded in a manner similar to *Lucilia sericata*	All legs lowered and extended forward	All legs lowered and extended forward
Diptera *Limoniinae* sp.	1st pair of legs held out in front, 2nd and 3rd pair stream out behind	1st pair of legs raised above head, 2nd pair brought forward	Legs lowered as with disk
Haematopota crassicornis *Empis livida* *Eristalis tenax* *Volucella pellucens* *Syrphus* sp. *Drosophila* sp. *Calliphora erythrocephala* *Pollenia rudis* *Muscina stabulans* *Musca domestica* *Dasyphora cyanella* *Stomoxys calcitrans* *Fannia anicularis*	Legs folded in a manner similar to *Lucilia sericata*	Legs lowered and extended forward	Legs lowered and extended forward

number of ommatidia stimulated, the log of the relative decrease of intensity by which they are stimulated and the rate at which they are stimulated. This type of stimulus may well be more effective for an insect such as *L. sericata* than one based on distance judgement with the legs lowered at a fixed distance from the object, since that would require a well-defined landing surface. As it is, the insect is equally readily stimulated to slow down and extend its legs ready to make contact when approaching well-defined landing surfaces, more or less uniformly shaded walls or ill-defined shadowed areas into which it is likely to be attracted by chemical stimuli. Waterhouse (1948) has shown that in general houseflies land upon the darkest surface available provided that they are not additionally stimulated in other ways. Observations upon *L. sericata* and *Calliphora erythrocephala* in the laboratory indicate that they also show a tendency to land in a shaded corner or upon the darkest wall of the cage. Nevertheless, *Lucilia* is fully capable of landing upon a light, or even a plain white wall. It is extremely likely, however, that even on approaching very light surfaces there are sufficient irregularities of illumination to provide the necessary stimulus. Observations on blind flies have shown that if the legs are not lowered as the flies approach a landing surface, as soon as the wing tips make contact with the surface in front of them as they sweep forward, the legs are extended and moved about until the surface is grasped. The movement is not so smooth as the normal landing reaction but it is effective and it is possible that flies encountering a completely evenly illuminated surface, which must be very rare, land on it in this way.

The fact that the perception of approach to a landing surface appears to be based on the multiplication of: the contrast presented to successive ommatidia, the number of ommatidia so stimulated and the rate of successive stimulation, would seem to support Hassenstein's claim (1951) that perception of movement in an insect's visual field is based upon the evaluation of the contrasts presented, by a process of multiplication in the central nervous system. The after-effect resulting from stimulation of one ommatidium is multiplied by the response of the subsequently stimulated ommatidium as the object moves across the visual field. The rate of stimulation is important in that the sooner the second ommatidium is stimulated the less will the after-effect of the first ommatidium have faded. When *Lucilia* is stimulated by a change of light intensity alone the response is still related to the product of $\theta_2 V_2 (I_3 - I_4)/I_3$. It may be that in perceiving changes of light intensity the central nervous system is comparing the previous intensity and the new intensity continuously by a process of multiplication.

SUMMARY

1. The leg movements and changes in the wing beat pattern which occur when the fly, *Lucilia sericata*, is in the proximity of a landing surface have been filmed and the sequence of events described in detail.

2. The legs are lowered from their retracted flight position as the result of a visual stimulus mediated by the compound eyes alone.

3. The moment at which the legs are lowered is not based upon an estimation by the fly of the distance between itself and the surface it is approaching. A normal landing response can be evoked merely by decreasing the light intensity of the surroundings without any movement occurring in the visual field.

4. The effective stimulus is based upon a multiplication of: the change of intensity at successive ommatidia when a fly approaches a landing surface, the number of ommatidia so stimulated and the rate of their successive stimulation. A given value of this product is required to evoke the landing response.

5. The landing responses of other Diptera and representatives of other insect orders have been described.

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STUDIES ON THE CHEMICAL COMPOSITION OF MUSCLE TISSUE

I. THE MUSCLES OF THE HAGFISH *MYXINE GLUTINOSA* L. AND THE ROMAN EEL *MURAENA HELENA* L.

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INTRODUCTION

This is the first of a short series of papers dealing with the composition of the muscles of some lower vertebrates and invertebrates, particularly from the standpoints of the osmotic concentration of various constituents and the accumulation or reduction of ions in muscle relative to those of the blood-plasma or serum. Satisfactory data on these aspects of muscle-blood equilibrium or steady state exist for only a few species, and are usually incomplete. Ideally, estimations of some fifteen or more major constituents have to be made and the concentrations expressed per kilogram water content. Then, it is desirable to differentiate between the intracellular and extracellular concentrations of various ions and compounds; this necessitates finding the volume of extracellular fluid (blood and interstitial fluid) in the muscle, and, knowing its composition to a greater or less degree, subtracting appropriate values from the figures for whole muscle to give the average concentrations in the muscle cells.

Total osmotic concentration of muscle may be obtained by micro-freezing-point determinations on single fibres or by vapour-pressure determinations on the 'juice' expressed from muscles; these may then be compared with similar determinations on the plasma. Correlation of such measurements with the sum of the analysed constituents expressed in mg. ions and mM (or milliosmoles) per kilogram water can be made. This is often difficult as a proportion of some of the constituents may be 'bound' to larger molecules such as proteins, exerting little osmotic effect, and the activities of ions in the water of the cells and of the plasma may be different.

While most progress has been made with cephalopod and crustacean muscle, to be described in other papers of this series (see Robertson, 1957, for a preliminary account of *Nephrops norvegicus* muscle), the present analyses of hagfish and Roman eel muscles are published because they show features of general interest, although they consist of inorganic analyses of whole muscle only. The cyclostome *Myxine glutinosa* L. is a primitive marine vertebrate whose blood is practically isosmotic with sea water, whereas *Muraena helena* L. is a marine teleost fish with a blood concentration only about one-third that of sea water (Robertson, 1954). As will be seen below there are considerable differences in composition between the muscles of the agnathan and the fish.

MATERIAL AND METHODS

Details of the collection of *Myxine* and *Muraena*, and the methods and results of analysis of the blood, have been given by Robertson (1954). Muscle tissue was obtained from the same specimens, after collection of the blood, by removing the skin and cutting off dorso-ventral strips of muscle close to the vertebral column. Care was taken to remove all visible connective tissue and, in the case of *Muraena*, small pieces of bone. The muscle was lightly blotted between sheets of filter-paper and separate weighed samples were used for the determination of cations (sodium, potassium, calcium and magnesium), chloride, sulphate, trichloracetic acid soluble inorganic and total phosphate, and water content.

Before deciding on the method finally adopted for analysis of the cations of the muscle, a comparison was made using cephalopod muscle of two dry-ashing methods, with and without sulphuric acid, and a method using a 5% trichloracetic acid filtrate. Conclusions from this study may be briefly enumerated. Dry ashing in a muffle furnace at dull red heat or below (about 600° C.), gave results identical with those obtained with a sulphated ash in some cases, in others somewhat lower (e.g. Na 92%, Mg 94%). Something in the trichloracetic extract inhibited the precipitation of sodium as sodium zinc uranyl acetate, and results were usually low for this ion unless the extract itself was evaporated down with sulphuric acid and ashed in the muffle furnace. Magnesium could be determined directly in the filtrate by the oxine method, but calcium results, even after two precipitations, were 140% high, owing presumably to contamination of the calcium oxalate with organic compounds which would react with the ceric sulphate used as oxidizing agent for the oxalate precipitate. Potassium estimations in the trichloracetic extract, using the silver cobaltinitrite method after evaporating suitable aliquots in the presence of sulphuric acid and dry ashing, gave similar results to those found in the two ashing techniques.

The method finally adopted for cations was to add 2–3 ml. concentrated sulphuric acid to 6–8 g. muscle (fresh weight) in a silica basin, or to the corresponding amount of dried muscle if the water content of the sample had been determined. The basin was then placed on a boiling water-bath until the muscle was reduced to a black tarry consistency, and then transferred to a muffle furnace which was allowed to heat from cold to dull red heat (about 600° C.). If too much carbon remained, the ash was carefully ground with a glass rod and heated again in the furnace after the addition of a few drops of sulphuric acid. The ash was dissolved in water and 0·3 ml. concentrated nitric acid with the aid of heat, and made up to 20 ml. volume. Suitable aliquots were used for the determination of cations according to the methods of Robertson & Webb (1939), with the minor modifications of precipitating magnesium in centrifuge tubes at 80–95° C., separating the hydroxyquinolate by centrifugation, and of removing phosphate before the sodium estimation. A synthetic salt solution resembling cephalopod muscle in inorganic composition was made up (in g./l. Na 1·5, K 4·2, Ca 0·12, Mg 0·49, PO_4 8·0), and it was confirmed that 1·5 mg. sodium could be determined in the presence of 4·2 mg.

potassium and 8·0 mg. phosphate within about 2% of the theoretical, when calcium hydroxide was used in the precipitation of phosphate from an aliquot of the solution, as suggested by Holmes & Kirk (1936).

Because of alleged deficiencies in the open Carius method of determining chloride in tissues (Heilbrunn & Hamilton, 1942; Shenk, 1954), comparisons were made between the method of Sunderman & Williams (1933) in which there is preliminary alkaline digestion of the muscle before the addition of silver nitrate and nitric acid, the open Carius method as given by Peters & Van Slyke (1932), and that of Wilde (1945) which uses muscle filtrates. Zinc hydroxide filtrates were prepared, however, with Somogyi's (1945) zinc sulphate-barium hydroxide reagents, and the chloride in the filtrates was determined by both the Volhard method and Sendroy's silver iodate method as used by Robertson & Webb (1939). On adjacent pieces of cephalopod (*Sepia*) muscle, all methods gave results within 1·5% of the mean, except the open Carius procedure in which the chloride value found was 4·4% lower than the mean after nearly 24 hr. digestion on a steam bath. Addition of sodium nitrate to the muscle filtrates as used by Wilde (1945) did not increase the amount of chloride found, nor did oxidizing agents such as potassium permanganate and hydrogen peroxide.

Since the yellowish colour of whole muscle digests in the technique of Sunderman & Williams was found to be very persistent and to cause end-point difficulties, the zinc hydroxide filtrates were preferred. Samples of 3–5 g. fresh weight were thoroughly ground with pure quartz sand in a porcelain mortar, together with 8–12 ml. Somogyi's 5% zinc sulphate solution, followed by the same quantity of 0·3 N-barium hydroxide solution. Extraction was allowed to proceed for 15–30 min. with frequent stirrings, and the solution filtered through a chloride-free paper. To aliquots of the filtrate in a boiling tube were added standard silver nitrate and 1 ml. nitric acid, and the tubes were kept in a boiling water-bath for a short time until the silver chloride was well coagulated. After cooling, the solution was filtered, the precipitate well washed, and the titration of the filtrate made with 0·5% ammonium thiocyanate solution, with a few drops of saturated solution of ferric alum as indicator. Each group of estimations was standardized using a control tube with the same volume of fluid, containing standard silver nitrate and nitric acid.

Sulphate was determined micro-gravimetrically as barium sulphate on a muscle filtrate prepared by grinding with quartz sand about 5 g. muscle with 15 ml. 5% mercuric chloride solution, and filtering after allowing several hours for extraction. To an accurately measured quantity of the filtrate was added 10 drops 2 N-hydrochloric acid and distilled water to about 50 ml. Three ml. M/20 solution of barium chloride were added, and after thorough stirring the precipitate was left to stand overnight. It was filtered through a porcelain or silica filter crucible and washed; the crucible was dried and ignited to remove the small amount of adsorbed organic matter, and then rewashed, dried and ignited.

The inorganic and total phosphorus in a 5% trichloracetic acid filtrate of muscle were estimated gravimetrically with oxine-molybdate solution (Berg, 1935). About 3 g. muscle was ground with 2·5 g. trichloracetic acid and the final concentration made up to 5% with distilled water; after overnight extraction the solution was filtered. Since the precipitation involves heating in an acid solution to 70° C., the 'inorganic' phosphorus probably contains true inorganic P, creatine phosphate P, and pyrophosphate P from adenosine triphosphate. Total phosphorus was obtained by ashing a portion of the filtrate with sulphuric acid and hydrogen peroxide.

Water contents were estimated by drying samples of fresh muscle to constant weight at 101° C. for 24–48 hr., and subtracting the dry weight from the fresh weight. Analyses could then be given in terms of concentration per kilogram water content.

RESULTS

The composition of Myxine *muscle and serum*

Three comparisons of the electrolyte composition of muscle and serum are shown in Table 1. The average osmotic concentration of *Myxine* serum calculated from the sum of the principal mg. ions comes to 98% of that of sea water, a finding published and discussed previously (Robertson, 1954). The inorganic ions and organic phosphates of muscle, however, reach a total of only 39–47% of the osmotic concentration of the same ions in serum. Only potassium and phosphorus are present in higher concentration in the muscle, the concentration factor of both compared to their values in the serum being about twelve.

Other features of the analyses are the low calcium content of the muscle and the relatively high sodium, which exceeds the potassium. This latter feature is unusual in muscle analysis, although it is found in some lamellibranch muscles (adductor muscles of *Mytilus*, slow adductor of *Pecten*; Potts, 1958), and holothurian muscle (Koizumi, 1935). If intracellular concentrations were calculated on the basis of a probable 10–15% extracellular space in the muscle, the potassium would exceed the sodium in *Myxine* muscle fibres.

Table 1. *Electrolyte composition of whole muscle and blood-serum of* Myxine glutinosa

	mg. ions/kg. water									Water (g./kg. or g./l.)
	Na	K	Ca	Mg	Cl	SO_4	'Inorg.' P	Total P	Total mg. ions	
1. Muscle	122	117·1	1·8	12·9	107	4·2	75·8	94·6	460	761
Serum	563	8·7	5·5	18·6	570	6·7	7·2	—	1180	923
2. Muscle	137	113·0	2·2	10·7	121	3·7	—	82·0	470	775
Serum	562	10·5	7·1	22·0	592	6·9	—	—	1201	920
3. Muscle	148	110·7	2·1	15·9	168	8·2	74·8	90·7	544	781
Serum	550	9·5	6·1	17·7	565	6·5	6·1	—	1161	920
Sea Water	506	10·8	11·1	57·8	592	30·5	—	—	1208	989

The figures for P are really mg. atoms. The mg. ions will therefore be slightly lower, since the adenosine triphosphate molecule has 3 atoms P.

Cation-anion balance as far as the analyses have gone is shown in Table 2. The chief difficulty here is in relating mg. ions phosphorus (strictly mg. atoms P) to

Table 2. *Cation-anion balance in* Myxine *muscle*

Ions	m-equiv./kg. water		
	Specimen 1	Specimen 2	Specimen 3
	Cations		
Sodium	122	137	148
Potassium	117	113	111
Calcium	3·5	4·4	4·2
Magnesium	25·7	21·5	31·8
Total	268	276	295
	Anions		
Chloride	107	121	168
Sulphate	8·5	7·3	16·3
Inorganic and organic phosphate mg. atoms P × 1·8	170	148	163
Total	286	276	347

milliequivalents, since no detailed fractionation of the organic phosphate compounds was attempted. The number of cations associated with 1 atom P at pH 7 is about 1·6 for orthophosphate, 2·03 for creatine phosphate, 1·94 for hexose monophosophate, and 1·3 for each P atom in adenosine triphosphate. In the rat, the average is about 2·21 m-equiv./1 mg. ion P, and 1·74 if related to 1 mg. atom P, the lower of the two figures taking into account the 3 atoms of P in the adenosine triphosphate molecule (calculated from Conway, 1950*b*). A factor of 1·8 has been chosen for the conversion in Table 2. Considering the uncertainty of this value applying to *Myxine* and the difficulties of analysis of muscle in general, the cation-anion balance as determined is satisfactory in the first two specimens, rather unsatisfactory in the third, where the chloride value is considerably higher than in the other specimens.

The composition of Muraena *muscle and plasma*

In contrast to *Myxine* in which the blood-serum is practically isosmotic with sea water (as is the blood of most marine invertebrates), *Muraena* has a plasma concentration about one-third that of sea water, all its ions being lower than those of the medium except bicarbonate and phosphate (Table 3). Whereas ions in the muscle of *Myxine* are only 39–47% of those in the blood, the analysed ions of

Table 3. *Electrolyte composition of whole muscle and blood-plasma of* Muraena helena

	mg. ions/kg. water									Water (g./kg. or g./l.)
	Na	K	Ca	Mg	Cl	SO_4	'Inorg.' P	Total P	Total mg. ions	
Muscle	25·0	165·2	9·3	7·4	23·7	0·8	63·6	76·6	308	768
Plasma	212	1·95	3·87	2·42	188	5·7	5·2	—	419·2*	917
Sea water	564	12·0	12·4	64·3	659	34·0	—	—	1346†	987

* Including NH_4^+, HCO_3^- and urea—430 mg. ions.

† Including HCO_3^-—1349 mg. ions.

Muraena muscle come to over 70% of those in the plasma, constituting about 70% of the osmotic concentration of muscle, which is assumed to be the same as that of the blood. Except for sodium, the cations in the muscle exceed those of the plasma, potassium especially being high, with a concentration factor of over eighty. Chloride and sulphate in the muscle are very low.

Table 4. *Cation-anion balance in* Muraena *muscle*

Cations	m-equiv./kg. water	Anions	m-equiv./kg. water
Sodium	25·0	Chloride	23·7
Potassium	165	Sulphate	1·5
Calcium	18·7	Inorganic and organic phosphate mg. atoms P × 1·8	138
Magnesium	14·9		
Total	224	Total	163

Cation-anion balance is shown in Table 4. The factor of 1·8 was again used for converting mg. atoms P to milliequivalents. A considerable deficiency in anions is apparent. Further samples of muscle were then analysed to eliminate if possible any doubt of serious analytical error. Two samples gave 153 and 163 mg. ions or milliequivalents potassium compared with 165 in Tables 3 and 4, itself a mean of duplicate estimations. Chloride in another sample came to 23·0 mg. ions or milliequivalents compared with 23·7 in the tables. Thus it may be inferred that considerable quantities of other anions are present; these will include lactate and bicarbonate. Binding of a certain proportion of some or all of the cations in un-ionized complexes is a possibility which has not been investigated.

DISCUSSION

Differences between total ionic concentrations in the bloods of *Myxine* and *Muraena* are associated with differences in total ionic content of their muscles: *Myxine* with 1181 mg. ions in the serum (98% of the sea water value) has an ionic content in the muscle of 491 mg. ions (41% that of sea water); *Muraena* with 419 mg. ions in its plasma (31% of the sea water value) has a muscle content of 308 mg. ions (23% of the ions in sea water). Except for its high sodium and low calcium values, *Myxine* muscle resembles muscles of marine invertebrates such as *Nephrops* (Robertson, 1957 and in preparation), rather than those of teleosts. The low calcium content of blood and muscle in the hagfish is correlated with the absence of calcification of the skeleton. The difficulty of removing completely small pieces of bone is a hazard in muscle analyses of teleosts, and higher values of calcium and magnesium than those of Table 3 were obtained from strips of muscle from the same specimen of *Muraena*, in which slight effervescence of the ashed material in the acid used for its solution indicated the inclusion of small fragments of bone.

Vinogradov (1953) has collected some previous analyses of the elementary composition of fish muscle, but these are not very suitable for comparison with the present data for several reasons: simultaneous plasma and muscle analyses were not made, the immediate history of the specimens prior to analysis is not known, and the viewpoint of most of the analysts was the nutritional value of the fishes. Thus, for example, Clements & Hutchinson's (1939) analyses of the ash constituents of Australian fish pertain to fish bought in the open market, and include skin with the muscle in unspecified instances.

Carteni & Aloj's (1934) analyses of the edible portions of four marine teleosts were made at Naples, and might be expected to be roughly comparable with the analysis of *Muraena*. It is obvious from a study of their data that the muscles must have contained pieces of bone, since the calcium varies from 48 to 195 m-equiv./kg. water, and magnesium from 25 to 60 m-equiv. Taking the sardine *Clupea* (= *Sardina*) *pilchardus* as an example, the total concentration of the ions sodium, potassium, calcium, magnesium, chloride and phosphate comes to 486 mg. ions/kg. water, cations (in the same order) in milliequivalents 53·8, 170·6, 152·1, 51·3 = 428, anions 65·6 + 189·3 = 255. Almost certainly some of the missing anions would be those of the carbonate and phosphate of bone, and the phosphate ion, deemed to be HPO_4^{2-} in their table, would be in part the trivalent phosphate of the bone salts. These presumed additions would tend to bring cation–anion balance closer. The relatively high sodium and chloride values in the four teleosts, 41–77 and 51–82 mg. ions, respectively, suggest the possibility of small amounts of sea water on the skin or muscle.

Included in McCance & Widdowson's (1940) extensive data on the chemical composition of foodstuffs are analyses of muscle fillets from four common teleosts including the haddock and plaice. From these it can be calculated that the total concentrations of the principal ions in three of the species range from 296 to 323 mg. ions/kg. water, with a mean value (308) similar to that of *Muraena*. Of individual ions, sodium is higher and potassium lower than in the *Muraena* analysis; chloride tends to be higher also (29–54 mg. ions). On the assumption of 1·8 as the factor for converting mg. atoms P to milliequivalents phosphate anions, cation–anion balance shows a 10, 34 and 42 m-equiv. deficiency in anions in three species, equality in the fourth.

Carteni & Aloj (1935) provide inorganic analyses of the edible portions of three elasmobranchs, and it is of some interest to compare these with the *Myxine* and *Muraena* analyses. Elasmobranch blood is more or less isosmotic with sea water, but unlike *Myxine* slightly less than half the osmotic concentration is due to salts, the remainder being due mainly to the presence of urea and trimethylamine oxide. From Carteni & Aloj's analyses of the edible portions of the blue shark (*Carcharinus*), smooth hound (*Mustelus*) and the sting ray (*Dasyatis*) can be calculated osmotic concentrations of 295, 302 and 428 mg. ions/kg. water, respectively, for the same ions as set out in Tables 1 and 3. In the first and third, sodium exceeds potassium as in *Myxine*. Deficiency in anions is 32, 138 and 11 m-equiv., respectively, although chloride is quite high, 108, 64 and 163 mg. ions. Thus elasmobranch muscle in the Italian analyses falls below that of *Muraena* in total ions in two of the species, and resembles that of *Myxine* in a fairly high sodium and chloride content. Bialaszewicz & Kupfer's (1936) analysis of the bases in dorsal muscle of the elasmobranch *Torpedo ocellata* gives a value of about 240 m-equiv./kg. fresh weight, slightly higher than those of the three *Myxine* analyses if these are expressed similarly (204, 214 and 230 m-equiv./kg.).

It has been known since the beginning of the century (Fredericq, 1904) that organic compounds must make up a large proportion of the osmotic constituents of muscle in marine invertebrates and elasmobranchs, and a small proportion in teleost fishes. In marine invertebrates these low molecular weight compounds include the organic phosphates acting as anions, such as arginine phosphate, adenosine triphosphate and the hexose phosphates, free amino acids (non-protein amino acids), trimethylamine oxide and glycine betaine (Robertson, 1957). In teleosts creatine phosphate replaces the arginine phosphate, and in addition to trimethylamine oxide and betaine are the dipeptides carnosine and anserine (Love, Lovern & Jones, 1959). Urea is especially abundant in elasmobranch muscle.

It is probable that free amino acids and trimethylamine oxide make up the major part of the 28% deficit in osmotic concentration of *Muraena* muscle (Table 3), and the 58% deficit in *Myxine* muscle (Table 1). Betaine may perhaps be present in *Myxine* muscle, as it has been found in the related lamprey *Lampetra fluviatilis* (Strack, Schwaneberg & Wannschaff, 1937).

Comparison of the absolute amounts and relative proportions of electrolytes and organic constituents in the muscles of *Myxine*, *Muraena* and the white rat *Rattus norvegicus* (Conway, 1950*a*) is made in Table 5. Organic compounds form a decreasing proportion of the osmotic constituents in the series from the cyclostome to the mammal (58–13%). Electrolytes in *Myxine* muscle average 180% those of the rat, in teleost muscle 113%.

Table 5. *Osmotic constituents of vertebrate muscle*

	mg. ions (mM)/kg. water	
	Inorganic ions and organic phosphates	Organic constituents
Cyclostomata *Myxine*	491 (42%)	690* (58%)
Osteichthyes *Muraena*	308 (72%)	111* (28%)
Mammalia *Rattus*†	273 (87%)	41 (13%)

( ) = % total osmotic concentration.

* Obtained by subtracting the figures in previous column from the total osmotic concentration, assuming the latter to be identical with that of the serum or plasma.

† Conway (1950*a*).

SUMMARY

1. Methods of analysis of muscle are discussed, particularly in regard to ashing and extraction procedures for inorganic ions and the determination of chloride.

2. Simultaneous analyses have been made of blood and muscle for the following ions: Na, K, Ca, Mg, Cl, SO_4, inorganic and total trichloracetic acid soluble P (inorganic and organic phosphates).

3. In *Myxine* muscle the ions analysed make up 39–47% (average 42%) of the total osmotic concentration, assuming it to be that of the blood-serum: 491 mg. ions/kg. water content compared with 1181 mg. ions/kg. water in the serum. The

latter is practically isosmotic with the surrounding sea water of 1208 mg. ions. Potassium and phosphate are the only ions higher in concentration in the muscle than in the serum, but the muscle concentration of sodium exceeds that of potassium. Low calcium concentrations of 1·8–2·2 mg. ions/kg. water in the muscle and 5·5–7·1 mg. ions in the blood serum, compared with 11·1 mg. ions in the sea water, are correlated with the absence of a calcified skeleton.

4. Muscle ions in *Muraena* come to 308 mg. ions/kg. water content, about 72% of the osmotic concentration of the plasma; the latter is about one-third that of the surrounding sea water. Potassium, calcium, magnesium and phosphate ions in muscle exceed their respective concentrations in the plasma, while the remaining sodium, chloride and sulphate ions are below the plasma values.

5. Cation–anion balance of the analysed constituents of *Myxine* muscle is satisfactory, but there is a considerable anion deficit in *Muraena* muscle, probably made up in part by lactate and bicarbonate ions.

The analytical work was done at the Marine Station, Millport and the Stazione Zoologica, Naples. I am indebted to the former Directors of these stations, Mr E. Ford and Dr Reinhardt Dohrn, respectively, for facilities and kindness. I am grateful for permission to occupy the British Association Table at Naples, and am especially indebted to the Carnegie Trustees for the Research Fellowship which enabled me to pursue this work.

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A RUBBER-LIKE PROTEIN IN INSECT CUTICLE

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The cuticle of arthropods is complex and varies enormously in structure, properties and composition even within the same species (cf. Richards, 1951). However, the mechanical behaviour is dominated by materials in the solid state of matter so that the mature cuticles hitherto described in the literature may be classed as *solid cuticle*. It was therefore surprising to find that certain patches of the exoskeleton in many insects exhibit long-range reversible elasticity of the rubber-like type. This paper describes three examples of *rubber-like cuticle* from locusts (Orthoptera) and dragonflies (Odonata). Similar structures have been found in all winged insects hitherto examined (Weis-Fogh, unpublished). Essentially the present survey is a qualitative analysis of the material basis for the rubber-like properties of three selected samples, based upon simple tests and techniques. The main conclusion is that the characteristic elasticity is caused by a peculiar protein, called *resilin*, which differs from other structural proteins also in respect of amino-acid composition (Bailey & Weis-Fogh, 1961). Resilin is insoluble in all solvents which do not break peptide bonds and since it is located as an integral part of the highly organized cuticle it is necessary to analyse the three samples in detail as to structure and many other properties. Only in this way is it permissible to conclude that the elasticity depends on only one type of substance and to characterize it in general terms.

From a theoretical point of view the demonstration of a true protein rubber may claim some interest not only because insects make use of it in the form of nearly perfect mechanical springs (Weis-Fogh, 1959) but particularly for two other reasons. First, even the best thermodynamic experiments on *elastin* are ambiguous as to the rubber-like nature of this protein because elastin shrinks when heated so that the internal energy seems to change a great deal with stretch rather than to remain constant (Meyer & Ferri, 1936; Wöhlisch, Weitnauer, Grüning & Rohrbach 1943). However, a number of quantitative tests proved beyond doubt that at least one protein, resilin, behaves as a true physical rubber (Weis-Fogh, 1961). These experiments were done on an elastic tendon from dragonflies described for the first time in this paper. Secondly, a true rubber consists of a three-dimensional network of molecular chains which are nearly free of one another, thermally agitated and randomly kinked, but which are fixed in the network by means of a few stable cross-linkages (cf. Treloar, 1958). A protein rubber may then serve as a stable deformable network in cells and tissues and it is possible that the so-called stroma proteins fall into this category although next to nothing is known about their nature.

RUBBER-LIKE CUTICLE

In the course of an anatomical investigation La Greca (1947) noticed some hyaline, elastic structures in the thoracic skeleton of grasshoppers; they are now known to play an important mechanical role in the flight machinery of locusts because they store kinetic energy from the wings as elastic energy during the upstroke and pay it back during the subsequent downstroke (Weis-Fogh, 1959). So far, they have not been investigated in any detail.

(a) *Three test pieces*

In the desert locust (*Schistocerca gregaria* Forskål) and in other grasshoppers there are two suitable samples, the *prealar arm* and the main *wing-hinge ligament.*

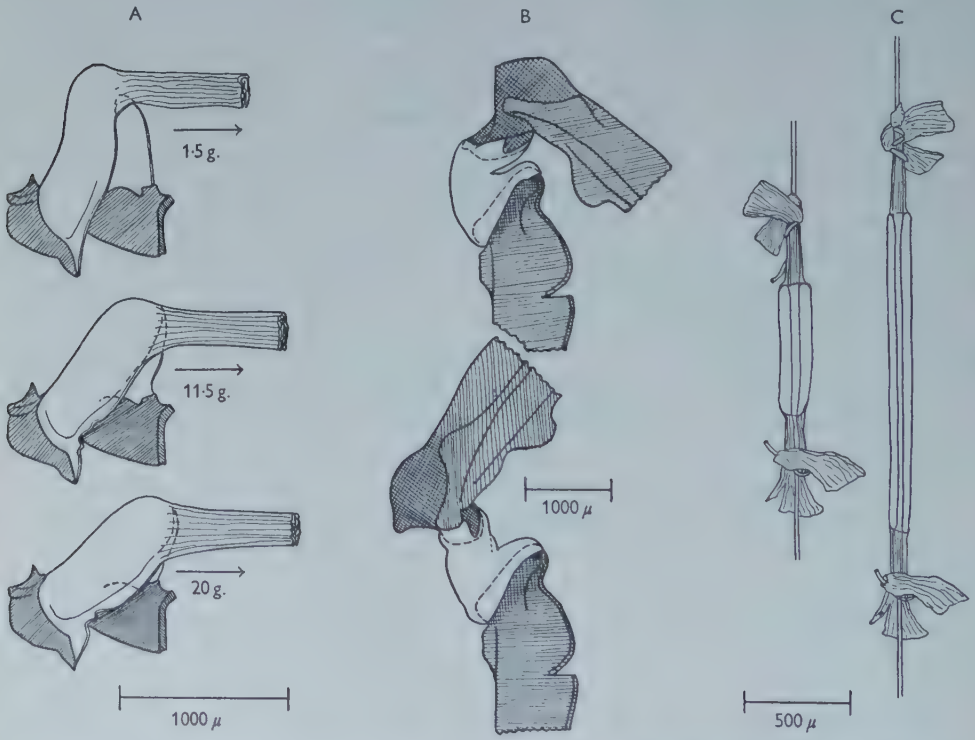

Fig. 1. Three examples of rubber-like cuticle. (A) The prealar arm from *Schistocerca gregaria*; the direction and magnitude of the load is indicated. (B) The main wing hinge of the forewing of *Schistocerca gregaria*; the resting position is shown at the top, and strained hinge at the bottom. (C) The elastic tendon of *Aeshna cyanea*, unstrained and extended. All cuticles were placed in dilute buffer at pH 7.

The prealar arm (Fig. 1 A) mediates the anterior suspension of the mesonotum, i.e. the movable plate between the forewings. It is a blunt conical peg about 0·5 mm. long which projects abruptly from a base of hard dark cuticle; at either side of the V-shaped groove it continues as ordinary tough arthrodial membrane (not shown). At the tip, the membrane is thickened to a tough flexible ligament which connects the prealar arm with the first basalar sclerite of the pleuron. A thread can be wound round the ligament and used in pulling experiments when the sclerotized base is embedded in plaster of Paris.

The wing-hinge ligament (Fig. 1 B) is a thick complicated cushion of hyaline rubbery cuticle which is inserted between the sclerotized pleural wing process and an equally hard and dark process on the underside of the second axillary wing sclerite (cross-hatched). The cushion represents the hinge proper and there is an exceedingly sharp and abrupt transition between it and the sclerotized parts although, of course, structural continuity is retained between the three elements of the hinge.

The third test piece is a peculiar sausage-like swelling of the tendon for the pleuro-subalar muscle in dragonflies (Fig. 1 C; muscles 34, 56; Clark, 1940). Clark (1940) mentions that the tendon feels elastic but the small swelling has escaped notice so far; in *Aeshna grandis* it is about 0·7 mm. long and 0·15 mm. wide. The entire tendon is built like the other cap tendons of this group, i.e. it is a deep hollow invagination from the dorsal body wall which widens at its ventral end to a cap-like insertion for a wing muscle. For the greater part of its length the tendon is tough and inextensible and only the hyaline swelling, the *elastic tendon*, is elastic like a rubber band.

Thin threads of nylon can be fastened to the tough parts for stretching experiments. The elastic tendon proper is a thick-walled, nearly cylindrical tube whose bore, here called the central canal, represents the lumen of the air-filled invagination. It has been found in all Odonata hitherto examined (*Uropetala carovei* Wh., *Libellula* spp., *Orthetrum* sp., *Sympetrum* spp., *Aeshna* spp., *Agrion* spp.). Tendons from the metathorax of *Aeshna cyanea* Müll. and *A. grandis* L. are almost cylindrical and suited for quantitative studies.

In Fig. 1 the three samples have been placed in saline at pH 7 and are shown unstrained as well as strained to the same extent as in the working animal. Even after great deformation it is characteristic that the elastic cuticle snaps back to the unstrained shape as soon as the load is removed. While solid cuticle breaks when stretched by 1 to 2% (Martin Jensen & Weis-Fogh, 1961), rubber-like cuticle is normally subject to deformations of 20–40%, exceeding 100% in the case of the tendon.

(*b*) *Appearance and water content*

It is easy to remove the thick hypodermal cells and their basement membrane and such stripped samples are transparent like glass and devoid of colour. The refractive index exceeds 1·4 and at pH 7 the water content is 50–60% (drying over P_2O_5, microbalance), in accordance with the swelling experiments illustrated in Fig. 8. Rubber-like cuticle is therefore a rather concentrated material.

The three samples all swell in alkaline buffers and shrink on the acid side of neutrality but they remain rubbery throughout. They become solid and glass-like only when dried in air or dehydrated in alcohol stronger than 90%. However, when water is again admitted they re-swell and become rubbery within seconds or minutes. Experiments like those illustrated in Fig. 2 may be repeated indefinitely with the same pieces of elastic tendon; in 70% alcohol they are completely rubbery, in 80% slightly plastic, in 90% they can be bent like plastic rods and deformed. If made hard in absolute alcohol while being strained to the extreme between a pair of forceps, the pieces quickly 'thaw' up in 70% alcohol and completely regain their original shape and size. This happens even after treatment with ordinary fixatives, protein coagulants and tanning agents (see p. 899) and also after embedding in paraffin wax or methacrylate. It is not usual to associate such properties with proteins.

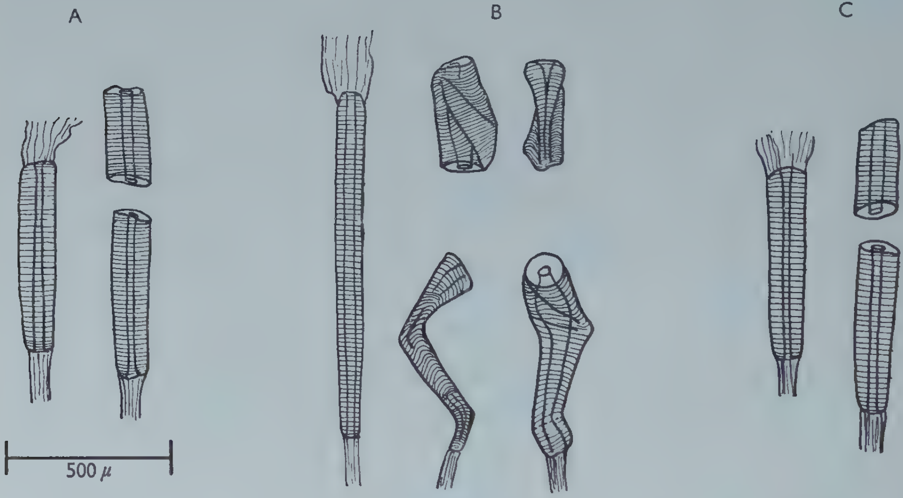

Fig. 2. A stripped elastic tendon and two cut pieces from *Aeshna grandis*. (A) Unstrained in 70% ethanol. (B) After extreme deformation and dehydration in absolute ethanol. (C) A short time after they were returned to 70% ethanol.

(*c*) *The elasticity is caused by protein*

It is, nevertheless, easy to prove that the characteristic elasticity resides with an insoluble protein, as is shown by the following experiments.

After incubation with papain at pH 7 or with other pure proteases (cf. p. 13), the prealar arm and the wing-hinge both became transformed into flaccid bags of thin lamellae. Although the external shape was retained, the rigidity and the elasticity had all gone and the cuticle appeared white and opaque due to light scattering by the refractile and glossy lamellae and the water between them. The delicate lamellae were continuous and could be separated by means of fine needles. They continued into the sclerotized cuticle (not affected) and into the membranes so that the pieces remained inextensible when stretched parallel to the lamellae. Similar results were obtained after mild alkaline or acid hydrolysis, such as 1 N–HCl at 65° C. for 5–6 hr. and 0·05 N-HCl or 0·05 N-NaOH for 1 hr. at 125° C. in sealed tubes.

Before hydrolysis slices of the prealar arm and of the wing hinge gave distinct Millon's and xanthoproteic reactions and stained blue with bromothymol blue followed by 5% acetic acid. After incubation about 80% of the dry matter had disappeared and the tests were negative. It was found that the hydrolysate contained many amino acids but neither sugars nor amino sugars; less than 2% of the original dry weight remained in the lamellar residue in the form of amino acids (Bailey & Weis-Fogh, 1961). The elastic material is therefore not a mucopolysaccharide or a glycoprotein, but seems to be a true protein which can be removed from the insoluble lamellae by proteases or mild hydrolysis.

The lamellae consist mainly (or exclusively?) of chitin. After treatment in saturated KOH at 160° C. for 15 min., all protein and elasticity had gone, but the lamellae were still present as glossy fragile sheets of the same shape and size as in the fresh cuticle. In all respects they reacted like chitosan (cf. Richards, 1951).

Most cuticles contain at least two protein fractions of which one is water-soluble and the other not. Prolonged treatment of thin slices from the prealar arm and the wing hinge with cold or hot (95° C.) buffered water failed to bring any significant amount of substance into solution (weighing). Moreover, a chromatographic analysis of the supernatant revealed that less than 0·3% of the protein goes into solution (Bailey & Weis-Fogh, 1961). Heating in water had no effect upon the elasticity of the three samples and, disregarding the thin epicuticle, there was no trace of lipid.

It is particularly instructive to consider the elastic tendon. After treatment with proteases, or with weak acids or bases, it appeared as an extremely delicate double tube. The central tube corresponded to the epicuticular lining round the central canal; the cortical tube was flush with the original surface of the stripped swelling and continued at either end as the 'external' layer (nearest to the hypoderm) of the tough cuticle. The space between the two tubes was emptied and filled with water and the tubes were so delicate that the structure was virtually deprived of strength in all directions. The intact tendon gave the usual protein reactions and the hydrolysate contained the same amino acids in approximately the same proportions as found in the two former preparations (Bailey & Weis-Fogh, 1961). Obviously, the entire strength and elasticity of the tendon is caused by a protein, both in compression and in extension. The cortical tube gave a faint but typical chitosan reaction, the tough ends and the transitional zone a massive reaction.

We may conclude that, disregarding the thin epicuticle, rubber-like cuticle owes its elasticity to a swollen water-insoluble protein. The only other structural component is sheeted chitin and the two components may be separated simply by means of mild hydrolysis.

STRUCTURE AND PROPERTIES OF RUBBER-LIKE CUTICLE

The complexity of ordinary solid cuticle is not found in the rubber-like samples and, knowing the arrangement of chitin and protein, it was therefore possible to analyse some fundamental properties of resilin from experiments with intact test pieces.

(*a*) *Some colour reactions*

Fresh or heat-treated rubber-like cuticle became sapphire blue in weak solutions of methylene blue or toluidine blue in dilute neutral buffers, 2–5 mg. dye per 100 ml. 0·05 M buffer. The colour was retained in pure buffer and is associated with the protein and not with the lamellae. This was particularly clear in the thick pad of elastic protein from the wing hinge which is described below and where there is no chitin at all. Toluidine blue stained orthochromatically so that the protein does not contain sulphate or metaphosphate to any appreciable extent (cf. Pearse, 1960). The colour reactions indicate that the isoelectric point is on the acid side of neutrality, in contrast to collagen, and it has been used to find rubber-like cuticles in a variety of other insects.

Fresh slices as well as paraffin sections of fixed rubber-like cuticle (Carnoy, Susa) did not stain with the periodic-acid-Schiff reagent (PAS), in contrast to the neighbouring tough ligaments and the arthrodial and basement membranes (cf. Wigglesworth, 1956). This is consistent with the absence of non-chitinous polysaccharide.

(*b*) *Prealar arm*

Structure. In transverse frozen sections of the prealar arm and its hypodermic cells, fixed in neutral formaldehyde, embedded in gelatin and plasticized in glycerol (cf. p. 12), the chitin lamellae form a concentric system of continuous refractile lines parallel to the hypoderm, each less than 0·2 μ thick. Nearest to the cells they are spaced apart by distances smaller than 1 μ and form a sort of 'skin'; further inside the interlamellar distance is 2–3 μ. In phase contrast and at the highest optical resolution the interlamellar substance is completely deprived of structure and is transparent like a gel. No trace has been found of pore canals and ducts from glands. In thin paraffin sections (4 μ) of material treated with Carnoy's and Susa's fixatives and embedded in balsam, the lamellae are distinct when stained with chlorazol black or light green (Fig. 3A). The interlamellar substance remains unstained and appears as an amorphous glass, often broken up into flakes by the passage of the knife but with no regular structure. It becomes deep red with Masson's and Mallory's triple stains where the green or blue lamellae are hardly visible unless in thin sections (4 μ; Fig. 3B). The red-staining material is not seen in the tough ligament nor in the arthrodial membrane but suddenly appears in the prealar arm proper; it is absent or not visible in the sclerotized part. It is also seen in the 'skin' which therefore differs from the remaining part only by a smaller distance between the lamellae. The hypodermal cells stain green for the first 1–2 μ nearest to the cuticle. After hydrolysis only the lamellae are present. The 'red' material is therefore considered identical with the elastic protein. It shows some affinity for Heidenhain's iron haematoxylin but, again, no microscopical structure.

The swollen protein acts as an elastic glue between the chitin lamellae which it separates by distances one order of magnitude greater than the thickness of the lamellae themselves. Thus the prealar arm is a simple multi-ply structure, and it is understandable that it behaves like a sample of pure rubber provided it is bent normal to the direction of the lamellae, as is the case in Fig. 1A and also in the experiments reviewed below.

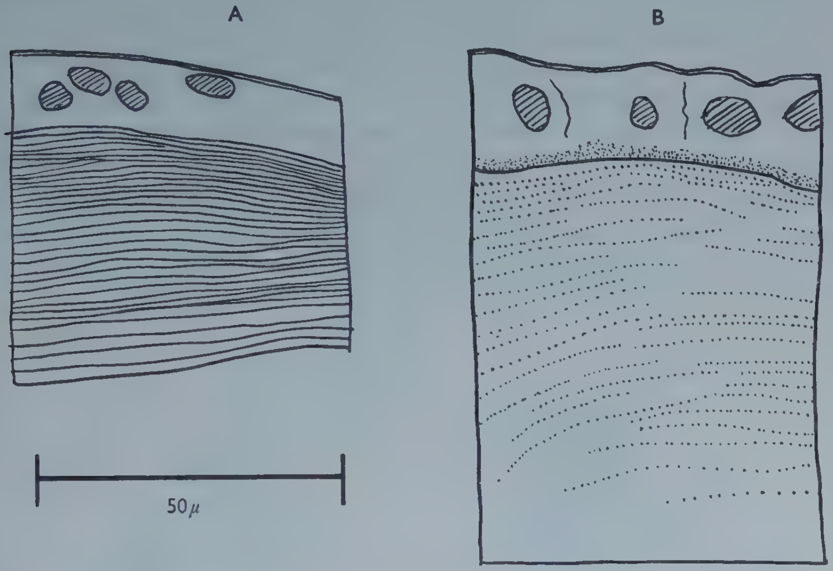

Fig. 3. Transverse paraffin sections (4μ) of the prealar arm, fixed in Carnoy's solution. (A) After staining with PAS and light green; only the green lamellae are seen. (B) After staining in Masson's triple stain; the green lamellae (indicated) are hardly seen through the opaque red protein. The cuticle-near border of the hypodermal cells is green (dotted).

Mechanical tests. In connexion with studies on locust flight, the prealar arm was tested during static and vibrational bending (Martin Jensen & Weis-Fogh, 1961). It turned out that the 'skin' had little effect in itself and that the rigidity is not due to turgor pressure but to the elastic properties of the entire structure, i.e. mainly to the protein. The static *modulus of elasticity* is about 20 kg. cm.$^{-2}$ ($2 \cdot 10^7$ dyn. cm.$^{-2}$) which is well within the range of true rubbers. Three additional results are also relevant here. After prolonged strain, the prealar arm recovers completely when the load is removed; the material therefore does not *flow*. The internal friction is astonishingly small since the *dynamic stiffness* increases by less than 5% compared with the static value and since the energy lost as heat per half cycle, the *loss factor*, amounts to less than 3% of the stored energy. These figures refer to speeds of deformation of the order of six sample lengths per second on the average.

Birefringence. True homogeneous rubbers are optically isotropic when unstrained but become birefringent on deformation. In frozen sections plasticized in glycerol and placed under the polarizing microscope, the protein between the lamellae could be seen to be isotropic provided the lamellae were viewed exactly from the edge. In most places and in thick transverse sections the swollen cuticle is positively birefringent in the direction of the lamellae; this is to be expected in a body where refractile laminae of chitin are embedded in a less refractile isotropic medium, as in a typical Wiener body. However, the birefringence could be modified and its sign reversed by swelling in alkaline media (pH 12). Since the chitin constrains swelling parallel to but not normal to the lamellae, the protein swells anisotropically and now it becomes positively birefringent in the direction of extension, i.e. normal to the lamellae, to such an extent that this intrinsic birefringence supersedes the structural birefringence and reverses the over-all sign. The changes are fully reversible.

When the prealar arm is mechanically compressed, the birefringence decreases and becomes negative in the direction of compression. A similar strain birefringence is also observed in extension: the structural birefringence was reduced by means of aqueous potassium mercury iodide (Thoulet's solution), the refractive index of which was adjusted to that of chitin (about 1·54); this solution causes the protein to swell and to extend normal to the lamellae, but does not dissolve it.

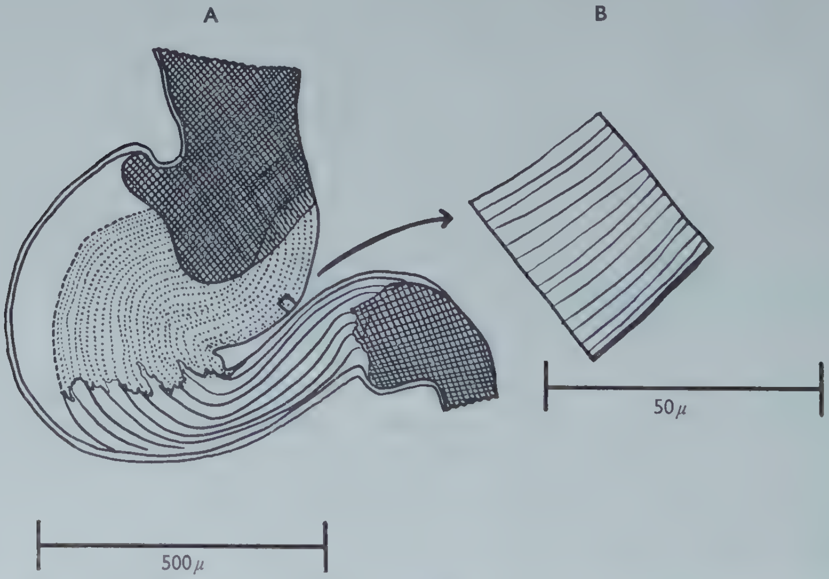

Fig. 4. Longitudinal frozen section of the wing hinge showing the sclerotized cuticle (double hatched), the tough flexible part (striped), the lamellar rubber-like part, and the pad of pure protein (resilin, white). (A) whole hinge; (B) part of lamellar cuticle, as indicated.

(c) *Wing hinge*

Mechanically the hinge shows the same elastic recovery and a similar small degree of damping as the prealar arm (Weis-Fogh, unpublished). The major part is also occupied by the same kind of lamellar cuticle (Fig. 4) but there are two differences. For a short length of the ligament, the ventral part is tough, dense and strongly birefringent when unstrained (striped). It contains protein which is not removed by hydrolysis in 1 N-HCl for 6 hr. at 65° C. since Millon's test was positive after hydrolysis; it appears as coarse fibrils densely packed between the chitin lamellae. The more interesting part is a *pad of elastic protein* up to 100 μ thick and situated between the hypoderm and the lamellar cuticle (white in Fig. 4). It has no microscopic structure whatsoever and is completely isotropic in polarized light. It can be broken up into irregular sharp-edged pieces; they remain optically isotropic at all degrees of swelling, and since they retain their relative proportions (within the accuracy of measurements, cf. p. 902) they are also mechanically isotropic; at equilibrium, the swelling forces must balance the elastic restoring forces. The swelling is reversible. The pieces exhibit typical strain birefringence, positive in the direction of extension.

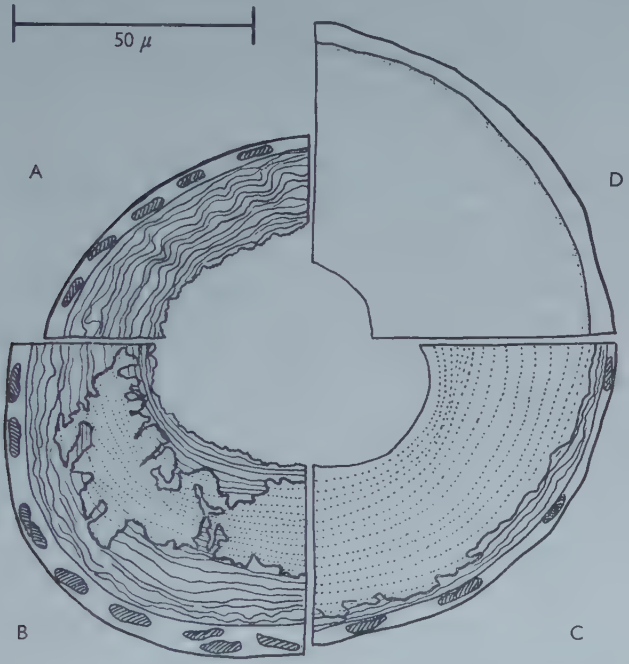

Fig. 5. Transverse paraffin sections (4 μ) of elastic tendon, fixed in Carnoy's solution and stained with Masson's triple stain. (A) Tough tendon dorsal to elastic part. (B) Transitional zone with first appearance of red-stained protein. (C) Lamellar rubber-like cuticle at anchoring zone. (D) The structureless belly with the red-staining protein filling up the entire space between epicuticle (centre) and hypoderm (periphery): the peripheral part stains less intensely (dotted).

(*d*) *Elastic tendon*

Structure. Pl. 17, fig. 1 shows the dehydrated mesothoracic tendon *in situ* (muscle no. 34, *Aeshna cyanea*, embedded in balsam). The hypoderm is just discernible; a glassy isotropic substance fills the broad space between it and the central canal. The cortical and central tubes are too thin to be seen, the dark outlines being optical artifacts due to refraction. In stained paraffin sections the tough invaginated membrane just dorsal to the swelling (Fig. 5A) appears like ordinary arthrodial membrane in *Periplaneta* (Dennell & Malek, 1954) and resembles the tendinous continuation at the ventral end. At the transition between membrane and swelling

(Fig. 5 B), green (Masson) or blue (Mallory) concentric lamellae dominate as in the former section, but an amorphous, strongly red substance makes its first appearance. The thin 0·2 μ pink epicuticle is thrown into wrinkled folds about 5 μ across, as in the membrane. A few microns further down (Fig. 5 C) the folds are absent and the membrane is densely supplied with papillae of a size close to the optical resolving power. The red substance has now spread over the entire cross-section apart from the cortical area and the appearance is like that of the prealar arm. However, still a few sections further down, the wall has attained its full thickness and the entire space between the papillose colourless epicuticle and the thin green cortical tube (0·2 μ or less) consists of a structureless solid stained red with Masson and Mallory and grey with iron haematoxylin. The outer 1–2 μ of the cortical zone is more faintly coloured with the two former stains and darker with iron haemotoxylin (Fig. 5 D). This must be the elastic protein which appears slightly different only towards the periphery but otherwise as in the locust. The cylindrical belly of the tendon is thus a thick-walled tube of pure protein, suitably anchored at both ends by a chitinous bell-shaped system of lamellae and contained between the delicate cortical and central tubes.

Simple mechanical tests. When the elastic tendon was stretched or compressed according to the methods described elsewhere (Weis-Fogh, 1961), the ultimate *deformability* amounted to 2·5 to 3 times the swollen length in extension (pH 7) and to 0·3 in compression, i.e. ten times variation in length in the direction of the axis. The diameter of the belly decreased uniformly with the degree of stretch both along the axis and in cross-section (cf. Pl. 17, fig. 2). The tensile *strength* was at least 30 kg. cm.$^{-2}$. When the belly broke, the surfaces were oriented almost normal to the axis and had a texture like that of broken flint (conchoidal fracture). After the break the two surfaces of the unstrained parts fitted exactly so that flow was absent even under these extreme conditions. When extended isotonically up to twice its previous length in neutral saline, *no flow* could be measured after days and even weeks of strain at room temperature; on release the tendon immediately snapped back to its original isotropic length within the degree of accuracy of estimation (0·5 %). The only difference was a faint residual birefringence, too small for measurement, next to the central canal after strains lasting for a week or more. It seemed to be connected with permanent deformations of the central tube and not of the rubber. After the break, the central tube sometimes projected from the surface; the cortical tube never came loose. The *elastic recovery* of the pure protein is therefore complete and instantaneous in the elastic tendon, in contrast to the prealar arm and the wing hinge where it takes some time, probably due to the chitin component (Martin Jensen & Weis-Fogh, 1961).

In extension the *elastic modulus* was estimated as about 6 kg. cm.$^{-2}$, allowance being made for the degree of swelling (cf. Treloar, 1958; Weis-Fogh, 1961). It was of the same magnitude in compression where neither the central nor the cortical tube could contribute to the elastic force. Taken together with the digestion experiments already described, the above results therefore apply to the rubber-like protein itself.

Birefringence. Pl. 17, fig. 2 shows a series of photomicrographs of the metathoracic tendon of *Aeshna cyanea* in neutral saline placed between crossed Nicols. The belly is completely isotropic at the unstrained length ($\lambda = 1$) and becomes increasingly birefringent, positive in the direction of extension, up to the greatest length ($\lambda = 2{\cdot}48$). In the last photograph, the relative darkening is due to the appearance of the first-order red interference colour. A dark band at either end marks the transition between the anchoring zone and the ordinary birefringent tendon. The unstrained belly and pieces cut from it were isotropic in all directions and at all degrees of swelling. The protein is therefore not constrained in any direction by materials behaving differently from itself. In highly swollen tendons (pH 12), there was sometimes a faint longitudinal striation not visible at ordinary degrees of swelling, but since it does not reflect any noticeable differences in strength or density it is justifiable to consider the protein as an isotropic rubber.

(*e*) *Conclusion*

Apart from the epicuticle, typical rubber-like cuticle consists of only two components both of which are insoluble and heat stable. The solid part consists of thin (0·2 μ) glossy lamellae of chitin which are spaced apart and glued together by thick continuous layers of an isotropic rubbery protein. In some places the protein is the only component of mechanical significance. Pore canals and other complicated structures or inclusions have never been observed.

The structural, optical and mechanical properties of the protein are basically alike in the three samples and correspond to those of a true rubber. Since, moreover, the reactions to water, dyes and chemicals seem identical, and since it has a unique amino-acid composition the protein component will be called *resilin* from now on (from Latin *resilire*, to spring back; Bailey & Weis-Fogh, 1961). It is further characterized in the next section.

GENERAL PROPERTIES OF RESILIN

Except where stated otherwise, the following tests were performed on slices cut from the three types of rubber-like cuticle just described.

(*a*) *Heat, coagulants, etc.*

In neutral water and in sealed tubes resilin does not dissolve and is not affected by heating up to 125° C. and it does not begin to disintegrate until between 140 and 150° C.

The following protein coagulants, tanning agents and fixatives all failed to bring about any permanent change from the rubbery to the solid state even after prolonged treatment, i.e. no histological or mechanical 'stabilization' is imposed: methanol, ethanol, acetone, 5 % acetic acid, 5 % $HgCl_2$, 1·5 % $K_2Cr_2O_7$, dilute as well as 40 % formaldehyde, saturated aqueous *p*-benzoquinone in phosphate buffer at pH 7·6 (cf. Gustavson, 1956), 1 % OsO_4 in neutral veronal buffer for 3 hr., Susa's, Bouin's (aqueous and alcoholic), and Carnoy's solutions.

When treated for 3 days at room temperature in aqueous 1 % CrO_3 with or without addition of sodium chloride and osmium tetroxyde (Flemming's solution), the strongly oxidizing reagent darkens the elastic tendon and causes it to shrink. The resilin is obviously degraded since it dissolves rapidly at pH 12. In fresh 10 % hypochlorite the *entire* tendon rapidly dissolves. If left for days in 1 % OsO_4, it goes pitch black by osmium deposits but still shows reversible swelling afterwards, although of reduced amplitude. When fixed according to Palade (1952) or Sjöstrand (1956), resilin attains only a light, smoky colour. It should be stressed that all aqueous solutions quickly and easily penetrate the rubber.

Isolated resilin slowly turns brownish when exposed to atmospheric air but its mechanical properties are not changed.

Some of the above reagents alter the swelling maximum apparently because they block various ionizable side groups (Martin Jensen & Weis-Fogh, 1961). This is, in fact, the only significant change that was observed as a result of treatments which profoundly alter most proteins. The secondary and tertiary structure of resilin must either be extremely stable or of little significance for rubberiness and stability.

(*b*) *Solvents, solutes and swelling agents*

Only polar solvents are of interest because non-polar solvents do not seem to penetrate at all. In water-free dioxan, for example, resilin shrinks by dehydration and remains glassy even at elevated temperature. A tendon was stretched in 70 % ethanol and dehydrated through the alcohol series. Its dimensions and strain birefringence remained constant in absolute methanol, ethanol, heptan, chloroform, benzene, benzyl alcohol, benzyl benzoate, α-bromonaphthalene, and methylene iodide. There is therefore hardly any chance of finding a non-polar embedding medium for sectioning which can penetrate and thus 'stabilize' resilin and similar proteins.

Some water-free polar solvents plasticize and swell resilin and make it rubbery, but none of them dissolve it. When a fresh piece is placed in glycol or glycerol, water is first removed by an osmotic effect and the sample becomes glassy before it swells again, starting from the outside. The following solvents act as plasticizers: ethylene glycol (slowly at room temperature, quickly at 60° C.), glycerol (starts at 60° C.), glacial formic acid (dissolves Nylon; loosens structure of collagen and keratin and makes them shrink, cf. Lloyd & Garrod, 1946), and concentrated formamide (rubberizes collagen). Several other plasticizers may undoubtedly be found.

Some aqueous solutes are known to break hydrogen bonds in proteins and to dissolve them but these had no effect on resilin: 6 molar urea, saturated guanidine hydrochloride, lithium chloride and iodide in various concentrations and temperatures. Thoulet's solution causes resilin to swell but does not dissolve it, as mentioned already.

Alkaline thioglycolate reduces the disulphide bonds of cystine and dissolves keratin and so does 6 molar urea plus 1 % thioglycolic acid, but resilin was resistant to these as well as to fresh performic acid which oxidizes the disulphide bridges.

Silk fibroin and cellulose are both held together by strong and numerous hydrogen bonds which are broken by alkaline cupric ethylenediamine without damage to the primary backbone (pH 12·4, prepared according to Coleman & Howitt, 1947). This reagent had no effect on resilin or chitin apart from the usual swelling at high pH.

Resilin is thus insoluble in spite of the fact that the molecular units are strongly solvated by water (hydration) and by other polar solvents, even when there is no net charge. It must therefore be cross-linked by very stable bonds or groups.

Fresh diaphanol (Gurr, 50% acetic acid saturated with chlorine dioxide) had an interesting effect. It is known to dissolve quinone-tanned proteins but not chitin in the course of a few days in darkness (cf. Brown, 1950). If a wing hinge such as that in Fig. 1 B is treated with diaphanol, the dark sclerotized cuticle becomes pale like amber in 3 days and is now softer than the rubber-like ligament which appears unchanged. The protein is therefore not cross-linked by quinones; but in the course of 10 or 12 days it slowly dissolves with the formation of a sticky glue, i.e. it probably breaks up into branched three-dimensional units rather than into linear molecules. The cross-linkages seem more resistant than the peptide backbone.

(*c*) *Digestion*

The insolubility of resilin is in marked contrast to the ease with which it is digested by all the proteolytic enzymes tested so far. Apart from non-crystalline papain at neutral pH and a 1% solution of the mixed proteolytic enzymes in the saliva of the reduviid bug *Platymerus rhadamanthus* at 28° C, and pH 7·5 (Soerensen's buffer, sample supplied by Mr J. S. Edwards, Cambridge), the following crystalline enzymes dissolved pure fresh resilin in the given order of speed; in lamellar cuticle the rate was smaller but eventually all resilin had gone: 1% porcine *pepsin* at 38° C. and pH 2·2 (0·1 M McIlvaine's buffer; L. Light and Co.), 0·5% *elastase* from pig pancreas at 37° C. and pH 8·9 (0·1 M Kolthoff's buffer, sample supplied by Dr M. A. Naughton, Cambridge), 0·5% salt-free *chymotrypsin* at 37° C. (0·1 M Kolthoff's buffer; Worthington), 0·5 and 1% *trypsin* at 38° C. and pH 7·5 (Kolthoff's buffer; salt-free trypsin from L. Light and $MgSO_4$-containing trypsin from Worthington).

Resilin is then easily digested, even by trypsin, and both in acid and alkaline media. The breakdown products are not known but the backbone must have many sites readily accessible to enzymes.

(*d*) *Reversible swelling and pH*

It is well known that the degree of swelling of a polyelectrolyte in water depends both on the solvating power and on the degree of ionization. The swelling increases with the number of ionized sites (cf. polyvinyl phosphate, Katchalsky & Eisenberg, 1950; polyglutamyl-lysyl-cysteine, Tani, Yuki, Sakakibara & Taki, 1953).

In dilute buffers, an amphoteric polyelectrolyte like resilin should therefore exhibit minimum of swelling at the isoelectric point. For this reason and in order to demonstrate the reversibility of the swelling phenomenon and the good correspondence between resilin samples from two different orders of insects (Odonata and Orthoptera), the following experiments were performed. Two almost rectangular pieces were cut from the fresh wing-hinge pad of *Schistocerca gregaria*, each containing about 2 μg. of dry matter. Similarly, two cylindrical pieces were prepared from the elastic tendon of *Aeshna grandis* (deep-frozen). The four pieces were placed on bronze gauze and simultaneously transferred to various dilute buffers (0·01–0·02 M; and 0·1 N-HCl), about 30 min. in each. Three characteristic lengths were measured microscopically as a set for each piece and used to calculate the volume relative to that of the air-dried sample placed in benzene. The accuracy is not great, ± 1–2% for relative length, so that the difference between the three lengths of one set could only be estimated to within ± 3% for each pad (found ± 4%) and ± 2% for each tendon (found ± 2%). But control observations in polarized light and the observed differences in relative lengths proved that the four samples swelled isotropically throughout the experiments summarized in Fig. 6.

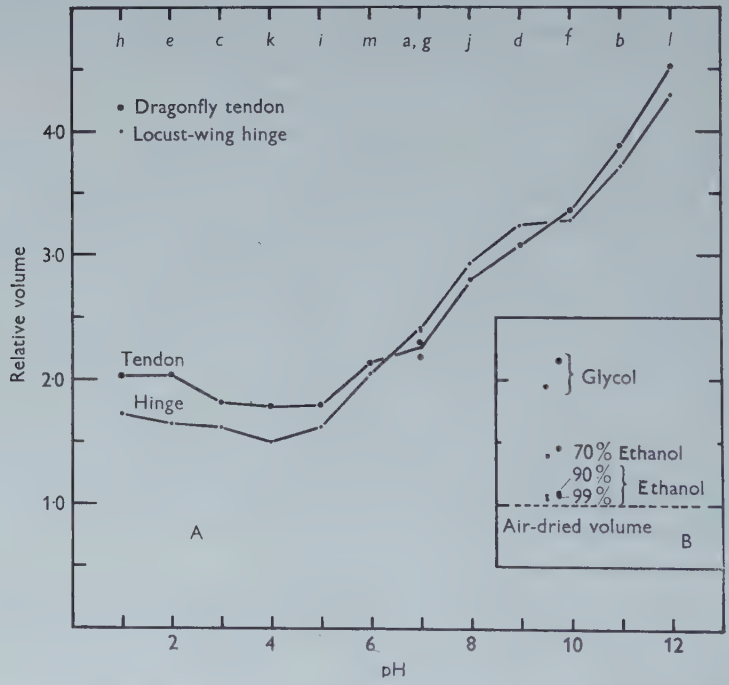

Fig. 6. Swelling and pH in pieces of pure resilin placed in dilute buffers (A). The lower-case letters indicate the sequence of transfers. (B) relative volumes in absolute glycol and various concentrations of aqueous ethanol. Ordinate: volume relative to volume of air-dried specimen. Abscissa: pH.

Each point is the average of two samples. The sequence of transfers is indicated by the lower-case letters at the top. Taken together with the smoothness of the curves, the results prove that the swelling of resilin is perfectly reversible, in conformity with the lack of mechanical flow and the chemically stable nature of the cross-linkages already referred to.

The two curves take almost the same course and both the tendon (filled circles) and the pad (dots) seem to have an isoelectric point about pH 4, although they are too flat in this region for accurate estimates. In fact, this flatness explains why the stiffness of the intact prealar arm changed so little from pH 1·8 to pH 5·6 compared with the changes at higher pH (Martin Jensen & Weis-Fogh, 1961).

Resilin from the two sources is then fundamentally alike in physical properties, but the results indicate that the pad may be less solvatable than the tendon; see also the inserted values from glycol and ethanol-water mixtures in Fig. 6B. However, this need not be so because it may only reflect a higher degree of cross-linking in the pad than in the tendon and therefore a higher resistance to swelling in the former, a result in accordance with the differences in elastic modulus.

DISCUSSION

According to modern views (see, for example, Treloar, 1958), a typical unstrained rubber consists of a three-dimensional network of randomly kinked and randomly oriented long-chain molecules. The actual chemical constitution is immaterial; the term 'rubber-like' describes a specific physical state of matter which is characterized in the following. The junction points of the network are either simple physical entanglements which are liable to flow or stable chemical cross-linkages. The chains must possess many freely rotating links between each pair of junction points and the secondary forces between the chains must be weak so that the average chain is randomly kinked as a result of thermal agitation, i.e. the configurational entropy of the rubber is maximum. The network owes its great deformability to the fact that the probable distance between the end points (junction points) of a randomly kinked chain is only a fraction of the extended chain length. Below a certain temperature, the rubber becomes rigid and solid like glass because the thermal movements are insufficient to overcome secondary intermolecular forces. By solvating the chains (by plasticizers) or by increasing the temperature the material resumes its rubberiness. Such a system is optically and mechanically isotropic in the glassy state, in the rubber-like state and at all degrees of swelling. When strained, the chains are removed from their statistically most probable configuration to which thermal agitation tends to bring them back. At the same time, the rubber becomes birefringent in the direction of the strain provided, of course, that the polarizability is different along the backbone and normal to it. At ordinary temperatures and at degrees of cross-linking compatible with a rubber-like behaviour, both theoretical and experimental estimates of the elastic modulus indicate a range from about 5 to 30 kg. cm.$^{-2}$. These figures are two to three orders of magnitude lower than found in most non-rubbery substances.

(*a*) *Resilin as a rubber*

All the evidence presented here strongly indicate that resilin is a true rubber and nothing speaks against this interpretation. However, its behaviour may result from a random network of protein chains which are regularly coiled and held in the coiled configuration by secondary forces, as is probably the case in keratin swollen with glacial formic acid (Lloyd & Garrod, 1946; 1948). The extensibility would then be caused partly by a lengthwise orientation of the network which could account for extensions up to twice the unstrained length and partly by an uncoiling of the chains. The latter mechanism means breaking of the secondary structure of the peptide backbone, and this is highly unlikely because we have seen that resilin is practically unaffected by reagents known to interfere strongly with the secondary structure of proteins (heat, coagulants, tanning agents, fixatives, agents breaking hydrogen bonds). Moreover, the elastic modulus is so low and the concentration of the material so high (40–50% dry matter at pH 7) that, in the case of uncoiling, the secondary structure of a chain should be broken by forces two magnitudes lower than what is necessary to transform α-keratin into the stretched β-configuration (cf. Bull, 1945). A rigid experimental proof for the rubber-like nature of resilin is provided elsewhere (Weis-Fogh, 1961).

(*b*) *Resilin, elastin and other proteins*

All evidence taken together strongly indicate that there are great physical and chemical similarities between resilin and elastin. Elastin is here considered as the homogeneous protein prepared according to Partridge, Davis & Adair (1955) from the *ligamentum nuchae* in the form of fragments of rather uniform fibrils, and not as the composite birefringent elastic tissue consisting of elastin, collagen, vitreous fibres and mucopolysaccharides (Hall, Reed & Tunbridge, 1952; Wood, 1954; Cruise, 1957; Romhányi, 1958).

In neutral water elastin and resilin are both rubber-like and swollen with about 50–60% of water, but they become rigid and glass-like on drying (McCartney, 1914). Lloyd & Garrod (1948) claim that elastin swells in methanol and ethanol, but the swelling is insignificant in resilin (Fig. 6B) which remains rigid at concentrations above 90%. On readmission of water, in aqueous alcohols and in many other polar solvents dried elastin and resilin re-swell isotropically and become rubbery, as in formamide and glacial formic and glacial acetic acid (Lloyd & Garrod, 1948). The isoelectric point of elastin is about pH 4 (Partridge, Davis & Adair, 1955) and, like resilin, it therefore swells in acid as well as in alkaline media. Neither is tanned by chromium salts (Roddy & O'Flaherty, 1939). Both are characteristically insoluble in all solvents and reagents which do not directly attack the peptide backbone (Lloyd & Garrod, 1948), including agents which reduce or oxidise disulphide groups (Wood, 1954); but they are digestible even by trypsin, according to Lloyd & Garrod (1948). They are heat stable and apparently not affected by neutral water up to 150° C.

It is therefore most likely that elastin and resilin share at least two important structural characteristics, a very limited tendency for the backbone to form secondary structures combined with a very stable kind of cross-linkage, the nature of which is unknown in both proteins (cf. Partridge & Davis, 1955). The swelling of resilin is the outcome of forces of solvation, of electrostatic repulsion between ionized groups and of Donnan phenomena.

Apart from these similarities and the large amount of glycyl residues in both, the amino-acid composition of resilin is very different from that of elastin (Bailey & Weis-Fogh, 1961) so that the ideas of Lloyd & Garrod (1948; cf. Kendrew, 1954, p. 948) concerning the conditions necessary for the establishment of rubberiness in proteins must be revised. Resilin is devoid of colour but it is not known whether the yellow pigment in elastin has any real significance or not (cf. Loomeijer, 1958).

Further chemical and physical analyses appear to be much simpler in resilin than in elastin, partly because resilin can be obtained as pure samples with no apparent structural or chemical impurities and partly because Nature has provided a neatly suspended pure sample of convenient shape, the elastic tendon.

(*c*) *On the formation of resilin*

It was observed that the large isotropic pad of resilin in the wing hinge of *Schistocerca* is deposited *after* the final moult and in the course of the first days of adult life. Since the pad does not contain carbohydrates or amino sugars, it is a particularly favourable object for studies on how the process of cross-linking is brought about and how organisms may synthesize three-dimensional networks. According to Smith (1957), the biosynthesis of elastin is virtually unknown.

(*d*) *Resilin and insect cuticle*

The demonstration of a new well-defined protein in insect cuticle is of interest to entomologists, particularly because it is clearly separated from chitin chemically and physically. The optical analysis has been confirmed by electron microscopy to be reported elsewhere. It has led to some new thoughts about the structure and morphogenesis of insect cuticle which are discussed in connexion with mechanical and functional aspects of solid and rubber-like cuticles (Martin Jensen & Weis-Fogh, 1961).

SUMMARY

1. A new type of hyaline, colourless cuticle, called rubber-like cuticle, is described and analysed qualitatively with respect to mechanical behaviour, structure and composition. Externally it is covered by ordinary thin epicuticle, but otherwise it represents the simplest type of cuticle known and consists only of thin continuous lamellae of chitin (0·2 μ) separated and glued together by an elastic protein, *resilin*, not hitherto described. There are only traces of water-soluble substances present and resilin sometimes occurs as pure, hyaline patches more than 100 μ thick and suitable for macroscopic experiments.

2. In all physical respects, resilin behaves like a swollen isotropic rubber but the rigid experimental proof is given elsewhere (Weis-Fogh, 1961). An outstanding feature is the complete lack of flow not paralleled by other natural or synthetic rubbers.

3. Resilin resembles elastin but it is devoid of colour and has a different and characteristic amino-acid composition (Bailey & Weis-Fogh, 1961). The nature of the cross-linkages is unknown at present but they are extremely stable, of a

co-valent type and different from other known cross-linkages in proteins. This accounts for its insolubility and resistance to all agents which do not break the peptide backbone.

4. Resilin is a structure protein in which the primary chains show little or no tendency to form secondary structures; they are bound together in a uniform three-dimensional network (the tertiary structure) with no potential limits as to size.

It is a pleasure to thank Sir James Gray, F.R.S., and Prof. V. B. Wigglesworth, F.R.S., for their help and hospitality during my years as a Balfour Student in the Department of Zoology, Cambridge. I have also profited much from discussions with Dr L. E. R. Picken, Dr M. G. M. Pryor and Dr O. Sten-Knudsen. The work was supported by grants from the Carlsberg Foundation, The Department of Scientific and Industrial Research and from The Rockefeller Foundation.

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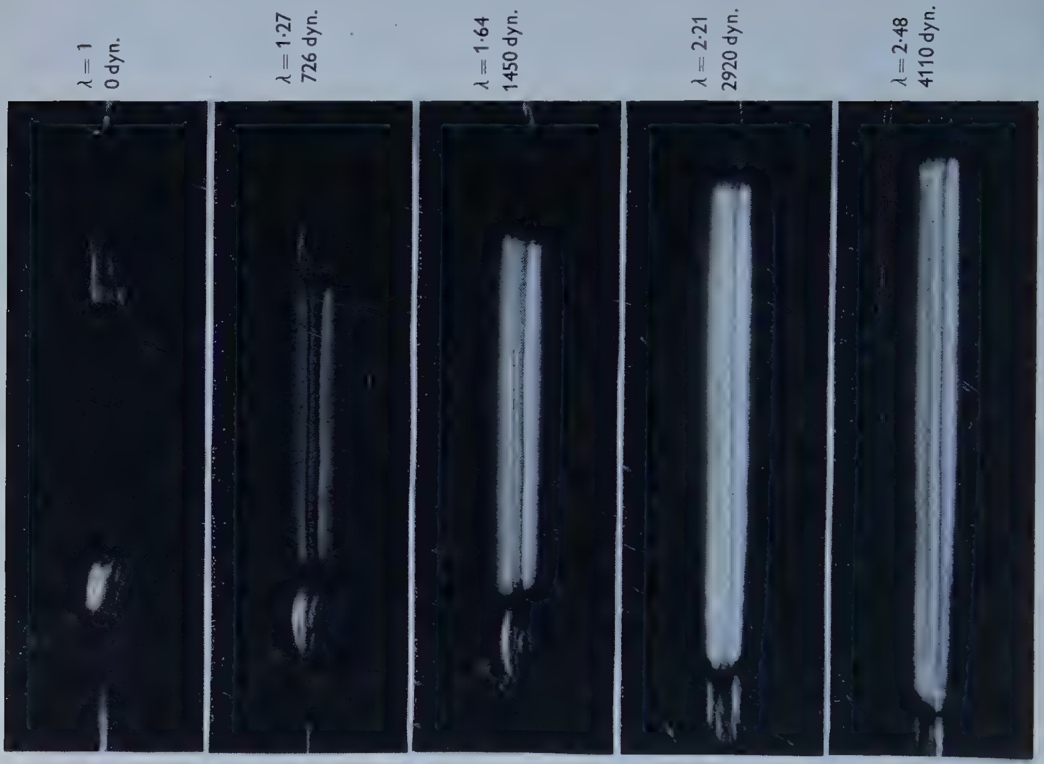

Fig. 2

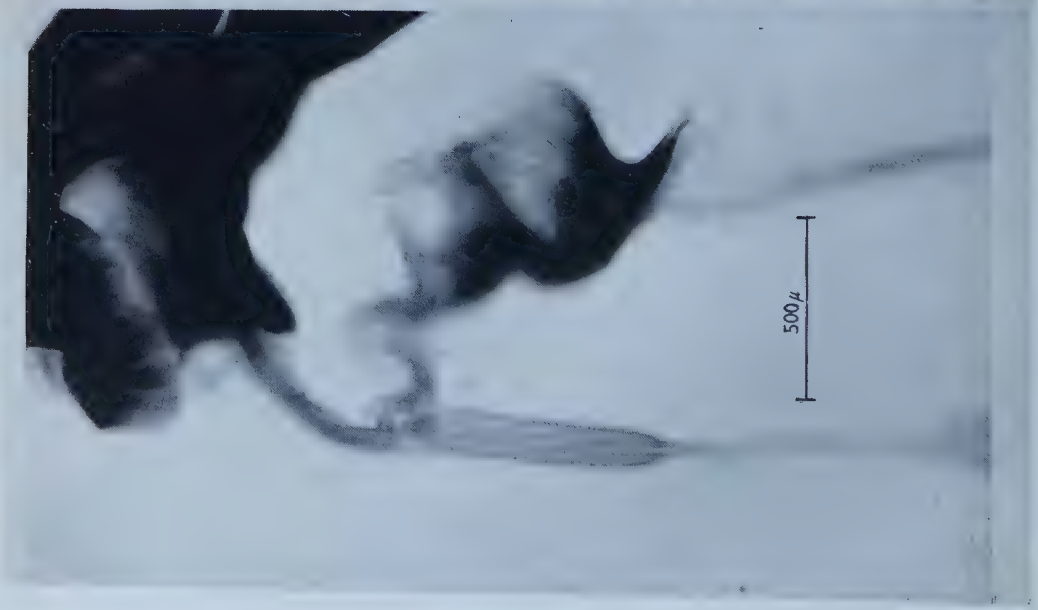

Fig. 1

TORKEL WEIS-FOGH—A RUBBER-LIKE PROTEIN IN INSECT CUTICLE

(*Facing p.* 907)

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EXPLANATION OF PLATE 17

Fig. 1. Elastic tendon from *Aeshna cyanea*, dehydrated and mounted in Canada balsam *in situ*. From the mesothorax.

Fig. 2. Fresh, stripped elastic tendon from *Aeshna grandis* placed between crossed Nicols both unstretched ($\lambda = 1$) and stretched up to $\lambda = 2{\cdot}48$. The tendon is birefringent only when stretched. From the metathorax. The loads are indicated (in dyn.).

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ABSTRACTS:

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